PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/15, C07K 13/00
G01N 33/574, 33/53, C12P 21/08
C07K 15/12, A61K 37/64, 49/02
A61K 47/48

A1

(11) International Publication Number: WO 91/09124

(43) International Publication Date: 27 June 1991 (27.06.91)

(21) International Application Number: PCT/AU90/00603

(22) International Filing Date: 20 December 1990 (20.12.90)

(30) Priority data:
PJ 7924 20 December 1989 (20.12.89) AU

(71) Applicant *(for all designated States except US)*: BIOTECH AUSTRALIA PTY. LIMITED [AU/AU]; 28 Barcoo Street, Roseville, NSW 2069 (AU).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : GOSS, Neil, Howard [AU/AU]; 118 Campbell Drive, Wahroonga, NSW 2076 (AU). RICHARDSON, Michael, Andrew [AU/AU]; 1 Benowra Place, Belrose, NSW 2085 (AU).

(74) Agent: GRIFFITH HACK & CO.; 71 York Street, Sydney, NSW 2000 (AU).

(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.

(54) Title: VARIANTS OF PAI-2

(57) Abstract

Variants of the plasminogen activator inhibitor PAI-2 in which the 66-98 amino acid residue region has been altered to eliminate at least one protease sensitive site are provided. The variants of the invention maintain the biological activity of PAI-2 and amino acids up to 65 and from 99 of PAI-2 in frame. The PAI-2 variants of the invention in labelled form, as well as DNA molecules encoding the variants of the invention, transformed host cells expressing the variants of the invention compositions and diagnostic kits comprising the variants of the invention, antibodies against the variants of the invention and processes for the production of the variants, DNA molecules, transformed hosts, compositions and antibodies of the invention are also described.

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VARIANTS OF PAI-2

TECHNICAL FIELD

This invention relates to genetically engineered variants of a plasminogen activator inhibitor, PAI-2.

DEPOSITION OF MICROORGANISMS

E. coli strain BTA 1445 was deposited with the American Type Culture Collection of 12301 Parklawn Drive, Rockville MD 20852, U.S.A. in accordance with the provisions of the Budapest Treaty under accession number ATCC 53585 on 11 February 1987.

BACKGROUND ART

Plasminogen activators (PAs) are serine proteases which convert the abundant extracellular zymogen, plasminogen, into plasmin, an active protease which can promote degradation of all components of the extracellular matrix. (Dano et al. Adv. Cancer Res. 44: 139-266, 1985).

Two different types of PAs have been recognised in mammalian tissues:

(1) Tissue-type Plasminogen Activator (t-PA).

t-PA is a serine protease with a molecular weight of about 70,000, composed of one polypeptide chain containing 527 amino acids. Upon limited digestion with plasmin the molecule is converted to a two-chain activator linked by one disulphide bond. This occurs by cleavage of the Arg 275 - Ile 276 peptide bond yielding a heavy chain (M_r 38,000) derived from the N-terminal part of the molecule and a light chain (M_r 32,000) comprising the COOH-terminal region. The catalytic site located in the light chain of t-PA is composed of His 322, Asp 371 and Ser 478. t-PA specifically catalyses the hydrolysis of an Arg 560 - Val 561 bond in plasminogen. Fibrin has been found to strongly stimulate plasminogen activation by t-PA.

(ii) Urokinase-type Plasminogen Activator (u-PA).

u-PA has an M_r of 50,000 and occurs in a one-polypeptide and a two polypeptide chain form. The one chain form is an inactive proenzyme, while the two-chain form is the active enzyme. u-PA has a substantial plasminogen activator activity in the absence of fibrin and is not stimulated by its presence. t-PA's high affinity for fibrin suggests that it is mostly associated with a fibrinolytic function while u-PA is associated with extracellular proteolytic events such as tissue remodelling and destruction (i.e. organ involution, inflammatory reactions and particularly in the invasive growth and metastatic spread of malignant tumours).

Experimental use of t-PA and single chain u-PA as thrombolytic agents in man has been promising. However, it has become apparent that PAs may have a less pronounced fibrin specificity in man than was anticipated from several animal models, suggesting a need for further improvement either of the agents or of their administrative schemes in clinical thrombolytic therapy. One possibility is the use of specific fast-acting protein inhibitors of PAs to modulate the systemic fibrinolytic effects of PAs.

Recent evidence suggests that urokinase-mediated plasminogen activation may also play a role in the invasive behaviour of malignant cells. With few exceptions malignant cells release PAs in abnormally high amounts. Ossowski and Reich (Cell 35: 611-619, 1983) reported that anti-urokinase antibodies inhibited the metastasis of human epidermoid carcinoma cells seeded onto chick embryo chorioallantoic membranes. Bergman et al (Proc. Natl. Acad. Sci. 83: 996-1000, 1986) have shown that protease nexin I, a fibroblast-secreted inhibitor of urokinase and plasmin, effectively inhibits the cell mediated degradation of extracellular matrix (ECM) by human fibrosarcoma (HT1080) cells. Finally, Sullivan and Quigley (Cell 45: 905-915, 1986) have demonstrated that a monoclonal antibody

to PA inhibits the degradation of ECM by Rous sarcoma virus-transformed chick fibroblasts. It follows from these observations and those of others [e.g. Mignatti et al., Cell 47: 487 (1986); Ossowski, Cell 52: 321 (1988); Reich et al, Cancer Res. 48: 3307 (1988)] that specific protease inhibitors of urokinase may play a critical role in altering the levels of active tumour cell PA in tumour tissue and therefore influence tumour growth and invasion in vivo.

There are other indications that a specific inhibitor of urokinase-type plasminogen activator has a role in modern medicine. PAs are involved in a range of inflammatory conditions such as arthritis. Plasmin can degrade cartilage [Lack, CH & Rogers, HJ (1958) Nature 182: 948] and low levels of fibrinolytic activity due to plasmin have been detected histochemically in synovial membranes. The PA/plasmin system has been detected in rheumatoid cell cultures [Werb, Z et al. (1977) New Engl J Med 296, 1017] and elevated levels of uPA have been noted in rheumatoid synovial fluid [Mochan, E. & Uhl, J. (1984) J. Rheumatol 11, 123]. Hence, the use of a specific inhibitor of uPA in arthritis could reverse the tissue destruction associated with this disease.

Other conditions where the application of a specific PA inhibitor may be of use include diseases or conditions such as osteoarthritis, multiple sclerosis, colitis ulcerosa, SLE-like disease, psoriasis, pemphigus, corneal ulcer, gastroduodenal ulcer, purpura, periodontitis, haemorrhage and muscular dystrophy. Finally, a PA inhibitor could have a significant role in skin wound healing and tissue repair especially since two trypsin inhibitors have been shown to enhance formation of connective tissue with increased tensile strength of the wound tissue [Kwaan, HC and Astrup, T (1969) Exp. Molec. Path. 11, 82] and keratinocytes are known to produce both uPA and tPA [Grondahl-Hansen, J et al. (1988) J. Invest Dermatol].

PA inhibitors, members of the serpin gene family (Sprengers and Kluft, Blood 69: 381-387, 1987), have been classified into four immunologically different groups:

1) Endothelial cell type inhibitor, PAI-1.
2) Placental type PA-inhibitor, PAI-2.
3) Urinary type PA-inhibitor, PAI-3.
4) Protease Nexin I, PNI.

PAI-2 (M_r about 46,000) has been purified from placental tissue, monocytes and the human monocytic cell line U937. The PAI-2 inhibitors from these different sources are immunologically related and recent cDNA sequence analyses of PAI-2 derived from human placenta and the human U937 cell line confirmed they are identical, although two forms of the molecule exist differing in only 3 single amino acid residues. Both cDNA forms have been isolated from U937 cells. (Schleuning et al. Mol. Cell. Biol. 7: 4564-4567, 1987; Antalis et al. Proc. Natl. Acad. Sci. 85: 985-989, 1988). PAI-2 reacts with both u-PA and t-PA (better with two chain t-PA than with single chain t-PA) to form SDS stable complexes. PAI-2 does not bind to fibrin or to fibrin-bound t-PA.

As is the case with most potent biologically active proteins, PAI-2 is produced in very small amounts in vivo and as such is difficult to purify and characterise by conventional biochemical approaches. The recent expression of PAI-2 in bacterial cells (Antalis et al. Proc. Natl. Acad. Sci. 85: 985-989, 1988; Bunn et al, Abstracts of the Second International Workshop on the Molec. and Cell. Biol. of Plasminogen Activation, Brookhaven National Lab., April 1989), now allows the production of quantities of purified PAI-2 needed to evaluate its biological efficacy in the various potential clinical applications described above.

DISCLOSURE OF THE INVENTION

Knowledge of the complete nucleotide sequence of PAI-2 allows specific genetic manipulations to be made which produce variants of PAI-2 which may exhibit improved properties compared with the native molecule.

Desirable improved properties include increased in vivo half life, increased or altered specificity, and/or improved pharmaceutical effectiveness.

The alterations may provide different properties which open up new areas of application or variants which are more amenable to industrial production, thus leading to improved production processes.

A unique difference between PAI-2 and the other serpins is the additional stretch of 33 residues (66-98) in the C_1-D interhelical region [Huber, R and Carrell RW (1989) Biochemistry 28 8951-8966]. This region is generally either limited to a short 9-residue stretch, as in ovalbumin or is absent, as in other members of the superfamily (eg human α1-antitrypsin, human antithrombin III). The significance of this structure is unknown. The present inventors have discovered that this region is sensitive to proteases, leading to the generation of a 37kD form of PAI-2 during production. The presence of a 37kD contaminant in PAI-2 preparations is not likely to be acceptable to regulatory authorities. Further, the 37kD form of PAI-2 is unable to bind to U-PA.

Thus it is desirable to provide biologically active PAI-2 molecules which are not sensitive to protease. When providing a variant of a particular protein which lacks an undesirable characteristic, it is not possible to predict whether the variant will maintain the desired biological activity of the parent protein, particularly where the alterations are significant. Given that the 66-98 amino acid region of PAI-2 is unique to PAI-2 it would be anticipated that alterations to this region of the molecule would be likely to render the resultant molecule inactive or at least have an adverse effect on its activity. Surprisingly, the variants of the present invention do retain the biological activity of native PAI-2, whilst lacking protease sensitivity.

Changes to PAI-2, can be made by modifying individual amino acids of PAI-2 by site-directed mutagenesis of the DNA or by wholesale restructuring by DNA

deletion or insertion to provide variants of the invention. The actual manipulations of the DNA can in general be performed in accordance with standard techniques in the art. The specific changes exemplified are produced by restructuring by DNA deletion.

According to a first embodiment of this invention there is provided a PAI-2 variant in which the 66-98 amino acid residue region of PAI-2 has been altered to eliminate at least one protease sensitive site which variant maintains biological activity of PAI-2 and amino acids up to 65 and from 99 of PAI-2 in frame. Preferably the variant is a deletion variant.

The invention particularly provides the PAI-2 variant Δ66-98 as herein defined wherein Δ66-98 has amino acids 66-98 inclusive of the PAI-2 amino acid sequence (SEQ ID NO.1) deleted. The invention also particularly provides the variant Δ74-96 as herein defined, wherein Δ74-96 has amino acids 74-96 inclusive of the PAI-2 amino acid sequence deleted.

According to a second embodiment of this invention, there is provided a PAI-2 variant of the first embodiment in labelled form.

According to a third embodiment of this invention there is provided a DNA molecule, the sequence of which encodes a PAI-2 variant of the first embodiment.

According to a fourth embodiment of this invention there is provided a recombinant DNA molecule comprising a DNA molecule of the third embodiment, and vector DNA.

Typically, the vector DNA is plasmid DNA.

Preferred plasmid vectors of the invention include E. coli expression vectors such as those based on the P_L promoter, lac promoter, tac promoter or trp promoter, pGEM4Z and vectors derived therefrom, pSp70 and vectors derived therefrom, baculovirus transfer vectors such as pAc373, pAc360 and vectors derived therefrom, mammalian expression vectors such as pBPV-1, pBPV-BV1, pdBPV-MMTneo, SV40 based expression vectors such as pBTA613, and vectors derived therefrom, vaccinia virus expression vectors,

retroviral expression vectors and other vectors used for the expression of recombinant DNA molecules in homologous or heterologous hosts.

Vectors derived from these vectors are those vectors obtained by making structural alterations to these vectors. Examples of the types of alteration include those made for the purpose of increasing expression from a particular vector.

pBTA613 is a mammalian cell expression vector. Foreign genes are expressed by cloning into the multiple cloning site flanked upstream by the SV40 early promoter and downstream by SV40 polyadenylation signals. pBTA613 comprises the following fragments in order. The 345bp PvuII-HindIII fragment from the SV40 origin, 51bp HindIII-EcoRI multiple cloning sites from pUC18, 75bp EcoRI-AatII fragment from pBR327, 853bp BamHI-XhoI fragment from pMSG with AatII linkers attached to both ends, 2262bp AatII-EagI fragment from pBR327, 27bp oligonucleotide (GGCCCATATGATATCTCGAGACTAGTC: SEQ ID NO. 4), 288bp EagI-SalI fragment from pBR327, 345bp PvuII-HindIII fragment from the SV40 origin, 734bp HindIII-BglII fragment encoding mouse dihydrofolate reductase from pSV2-DHFR, 141bp Sau3A fragment from SV40 small t intron region and 293bp Sau3A fragment from SV40 early polyadenylation region. The HindIII site at the 5' end of the dhfr gene was deleted using S1 nuclease, other incompatible ends were made flush using S1 nuclease or filled in with dNTPs and DNA polymerase I (Klenow).

Preferred recombinant DNA molecules of the invention include pBTA829, pBTA840, pMINDEL 74-96, and derivatives of these recombinant DNA molecules.

Derivatives of these recombinant DNA molecules are molecules derived from these molecules and include molecules where alterations have been made to the DNA structure for purposes such as improving or altering the control of expression of the encoded PAI-2 variant. The recombinant DNA molecule derivatives of the invention maintain the PAI-2 variant coding region of the parent molecule.

According to a fifth embodiment of this invention there is provided a transformed host cell transformed by a recombinant DNA molecule of the fourth embodiment.

Typically, host cell lines are derived from suitable E. coli K-12 strains. They can also be derived from eukaryotic organisms, and can include COS cells, CHO cells, U937 cells, BHK-21 cells, Vero cells, CV1 cells, C127 cells and cell lines derived from the insects Spodoptera frugiperda and Bombyx mori.

According to a sixth embodiment of this invention there is provided a process for producing a PAI-2 variant of the first embodiment, which process comprises: deleting nucleotides from the 66-98 amino acid residue region of a DNA molecule encoding PAI-2 such that the amino acids up to 65 and from 99 remain in frame and the resulting variant maintains the biological activity of PAI-2.

According to a seventh embodiment of this invention there is provided a process for producing a recombinant DNA molecule of the fourth embodiment, which process comprises inserting a DNA molecule of the third embodiment into vector DNA.

According to an eighth embodiment of this invention there is provided a process for producing a transformed host cell of the fifth embodiment, which process comprises making a suitable host cell competent for transformation, and transforming the competent host cell with a recombinant DNA molecule of the fourth embodiment.

According to a ninth embodiment of this invention there is provided a therapeutic and/or a diagnostic composition comprising an effective amount of at least one PAI-2 variant of the first embodiment together with a pharmaceutically acceptable carrier, excipient and/or diluent. The pharmaceutically acceptable carriers, diluents and excipients which may be used can be selected from those standardly used in the preparation of pharmaceutical formulations. When used for diagnostic purposes the agent may comprise the at least one variant in labelled form. PAI-2 variants may be labelled with a

radioisotope such as I^{131} or conjugated to an appropriate enzyme or other chemical agent. Particularly provided are such agents wherein the at least one variant comprises Δ66-98 and/or Δ74-96, as herein defined. When used for the production of antibodies the composition may comprise an adjuvant.

According to a tenth embodiment of this invention there is provided a method of inhibiting tumour invasion and/or treating tumours comprising administering to a patient requiring such treatment a therapeutically effective amount of a PAI-2 variant of the first embodiment and/or a composition of the ninth embodiment.

According to an eleventh embodiment of this invention there is provided a method of treatment of an inflammatory disease such as rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, ulcerative colitis, psoriasis or pemphigus comprising administering to a patient requiring such treatment a therapeutically effective amount of a PAI-2 variant of the first embodiment and/or a composition of the ninth embodiment.

According to a twelfth embodiment of this invention there is provided a method of treatment of a fibrinolytic disorder, such as systemic fibrinolysis, comprising administering to a patient requiring such treatment a therapeutically effective amount of a PAI-2 variant of the first embodiment and/or a composition of the ninth embodiment.

According to a thirteenth embodiment of this invention there is provided a method of treatment of a condition such as multiple sclerosis, corneal or gastroduodenal ulceration, purpura, periodontitis, haemorrhage or muscular dystrophy, comprising administering to a patient requiring such treatment a therapeutically effective amount of a PAI-2 variant of the first embodiment and/or a composition of the ninth embodiment.

According to a fourteenth embodiment of this invention there is provided a method for locating and/or defining the boundaries of a tumour in a histological

specimen or in vivo which method comprises applying an effective amount of a labelled PAI-2 variant of the second embodiment to the specimen or administering it to a host in need of in vivo imaging and determining by imaging, location of concentration of the label.

According to a fifteenth embodiment of this invention there is provided a method of improving the clinical efficacy of PA treatment of thrombosis which method comprises administering a therapeutically effective amount of a PAI-2 variant of the first embodiment and/or a composition of the ninth embodiment to a host in need of such treatment to counteract systemic activation of fibrinolysis and concomitant fibrin/fibrinogen breakdown.

According to a sixteenth embodiment of this invention there is provided an antibody against a PAI-2 variant of the first embodiment. The antibody may be either a monoclonal or a polyclonal antibody.

The antibodies of the present invention can be used for detecting PAI-2 and hence should be useful in the detection or monitoring of a number of disease states or conditions such as monocytic leukaemia, cancer, foetal development and chronic inflammatory diseases.

According to a seventeenth embodiment of this invention there is provided a process for preparing an antibody of the sixteenth embodiment, which process comprises immunizing an immunocompetent host with an effective amount of a PAI-2 variant of the first embodiment and/or a composition of the ninth embodiment.

According to an eighteenth embodiment of this invention there is provided an antibody composition comprising an antibody of the sixteenth embodiment together with a pharmaceutically acceptable carrier, diluent and/or excipient.

The antibody composition of the invention is of use in the detection or monitoring of disease states or conditions for which the antibodies of the sixteenth embodiment can be used.

According to a nineteenth embodiment of this invention, there is provided a diagnostic reagent comprising an antibody of the sixteenth embodiment and/or an antibody composition of the eighteenth embodiment.

According to a twentieth embodiment of this invention there is provided a conjugate comprising a variant of the first embodiment linked to a cytotoxin. Examples of cytotoxins which may be used in the preparation of conjugates of the invention include: abrin; ricin; mellitin; gelonin; and the A sub unit from Diphtheria, tetanus, clostridial, Pertussis, Shigella, Pseudomonas, cholera or E. coli labile toxin.

Previous studies have demonstrated that human colon cancers produce significantly greater amount of urokinase-type plasminogen activator than that occurring in adjacent non-involved tissue. PAI-2 has been found to be capable of binding to and inhibiting this tumour associated plasminogen activator (Stephens et al. Blood 66 333-337, 1985). Thus, it follows that biologically active PAI-2 variants have application as reagents for identifying and defining tumours both in vivo and in histological specimens. For imaging tumours in vivo PAI-2 variants of the invention may be labelled with an appropriate isotope, such as Technetium-99m (Richardson, V.J. Brit. J. Cancer 40; 35, 1979) or Iodine-131 (Begent, R.H.J. Lancet, Oct 2. 1982). Following administration of the PAI-2 variant preparation, the location and boundaries of the tumour may be determined by known radioisotopic methods, such as gamma-camera imaging. Thus, PAI-2 variants offer a sensitive method for enabling the identification of small metastic cancers particularly those arising after surgical intervention. In the analysis of histochemical specimens, PAI-2 variants or antibodies raised thereto, may be labelled with an isotope such as I^{131} or conjugated to an appropriate enzyme or other chemical reagent. On contact with a histological specimen, such as a biopsy section, a PAI-2 variant of the invention will bind to the tumour type plasminogen activator at its place of secretion, thereby

identifying the tumour boundaries and potentially the metastatic state of the tumour. In addition to diagnostic applications, PAI-2 variants are also indicated for use in the direct treatment of tumours. As specific inhibitors of the enzyme implicated in the process by which tumors invade surrounding tissues (Dano, K. et al., Adv. in Cancer Res. 44, 139, 1985), regulation and in particular, inhibition of tumour growth and metastases can be achieved. Furthermore, PAI-2 variants can be used as a drug delivery system to deliver lectins or toxins directly to growing tumours. It will be appreciated that this system could offer many advantages in terms of specificity and extremely potent tumouricidal capability.

Other biological processes in which urokinase-type plasminogen activators have been implicated involve those physiological events associated with invasion and tissue destruction, such as chronic inflammatory conditions including rheumatoid arthritis. PAI-2 variants are indicated to have a therapeutic effect when administered in vivo in ameliorating such conditions.

According to a twenty-first embodiment of this invention there is provided a cytotoxic composition comprising a conjugate of the twentieth embodiment together with a pharmaceutically acceptable carrier, diluent and/or excipient.

According to a twenty-second embodiment of this invention, there is provided a method of delivering a cytotoxic agent to a tumour which method comprises administering an effective amount of a conjugate of the twentieth embodiment, and/or a cytotoxic composition of the twenty-first embodiment to a host in need of such treatment.

According to a twenty-third embodiment of this invention there is provided a diagnostic kit comprising a variant of the first embodiment and/or a composition of the ninth embodiment as a standard and an antibody of the sixteenth embodiment, an antibody composition of the eighteenth embodiment and/or a diagnostic reagent of the nineteenth embodiment.

The diagnostic kits of the invention are of use in the detection or monitoring of diseases and conditions for which the antibodies of the invention can be used.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 PAI-2 cDNA and amino acid sequence (SEQ ID NO. 1). The arrows indicate the specific cleavage points so far identified.

Fig. 2 Bacterial (pBTA641) and baculovirus transfer vector (pAc373) used in expression of PAI-2 and its variants in E. coli K-12 and insect cells respectively.

Fig. 3 Immunological detection of PAI-2 derived from uninduced (lanes 1 and 2) and induced (lanes 3 and 4) E. coli K-12 cells containing plasmid pBTA641.

Fig. 4 Specific nucleotide and amino acid changes within PAI-2 to create the deletion variants Δ66-98 (SEQ ID NO. 3) and Δ74-96 (SEQ ID NO. 2). The altered regions are described in: SEQ ID NO. 5 for 66-98; SEQ ID NO. 6 for 74-96; and compared with the native sequence in SEQ ID NO. 18.

Fig. 5 Sequence of oligonucleotides A1 (SEQ ID NO. 7)and A2 (SEQ ID NO. 8), used to create the deletion variant Δ74-96 in pBTA829.

Fig. 6 Construction of plasmid pBTA829 containing the deletion variant, Δ74-96. (Abbreviations used: B, BglII; H, HinfI; P, PstI; E, EcoRI; P_L, leftwards promoter of bacteriophage lambda; T_7, promoter from bacteriophage T_7; Ap, ampicillin resistance gene; CIAP, calf intestine alkaline phosphatase)

Fig. 7 Schematic representation of PAI-2 specific bands (Fig 7A) and urokinase specific bands (Fig 7B) detected in a u-PA binding experiment.

Fig. 8 Sequence of oligonucleotides A134/301 (SEQ ID NO. 9), A134/304 (SEQ ID NO. 10) and A134/305 (SEQ ID NO. 11), used to create the deletion variant Δ66-98 in pBTA840. The location of the oligonucleotides within the PAI-2 coding region is

indicated by the accompanying numbers. The numbering of the bases is as in Figure 1.

Oligos A134/301 and A134/305 were used in a PCR reaction to generate a DNA fragment spanning the PAI-2 coding region from bases 235 to 541, with bases 244 to 342 inclusive deleted.
Oligos A134/304 and the Sp6 sequencing primer were used in a PCR reaction to generate a DNA fragment spanning the PAI-2 coding region from bases 49 to 351, with bases 244 to 342 inclusive deleted.
Oligos A134/301 and the Sp6 sequencing primer were used in a PCR reaction containing the products of the above two PCR reactions to generate a DNA fragment spanning the PAI-2 coding region from bases 49 to 541, with bases 244 to 342 inclusive deleted.

Fig. 9 Construction of plasmid pBTA840 containing the deletion variant, Δ66-98. (Abbreviations used: B, Bgl II; E, EcoRI; P, Pst I; P_L, leftwards promoter of bacteriophage lambda; Sp6, promoter from bacteriophage Sp6; T_7, promoter from bacteriophage T_7; Ap, ampicillin resistance gene; CIAP, calf intestine alkaline phosphatase; PCR, polymerase chain reaction).

Fig. 10 SDS-PAGE analysis of purified PAI-2 Δ66-98. 12% polyacrylamide gel (a.) and western (b.) (Anti-PAI-2 polyclonal antibodies) of the PAI-2 deletion mutant 66-98.

Fig. 11 Schematic representation of PAI-2 specific bands detected in a binding experiment with U-PA, two chain t-PA and single chain t-PA.

BEST MODE FOR CARRYING OUT THE INVENTION

The recombinant DNA molecules and transformed hosts of the invention are prepared using standard techniques of molecular biology.

Variants of the invention are obtained by culturing the transformed hosts of the invention under standard conditions as appropriate to the particular host and separating the variant from the culture by standard techniques. The variants may be used in impure form or may be purified.

Changes to PAI-2 can be made by modifying individual amino acids of PAI-2 by site-directed mutagenesis of the DNA or by wholesale restructuring by DNA deletion or insertion. These changes can be accomplished in a variety of ways well known to those skilled in the art [e.g. "Molecular Cloning, A Laboratory Manual" Chapter 15 "Site-directed Mutagenesis of cloned DNA" J. Sambrook, E.F. Fritsch, T. Maniatis (eds) 1989; "Current Protocols in Molecular Biology" Chapter 8 "Mutagenesis of Cloned DNA" Ausubel, Brent, Kingston, Moore, Seidman, Smith and Struhl (eds) 1989], and include the use of oligonucleotides for point insertion and deletion mutagenesis, degenerate oligonucleotides for nested mutations , the combing of long oligonucleotides to create a gene or gene segment with any desired changes, the use of Bal 31, DNAase I or Exonuclease III to create deletion mutants, the use of chemicals and the use of polymerase chain reaction (PCR). These techniques can be used to alter the 66-98 amino acid residue region of the PAI-2 molecule to produce PAI-2 variants. The PAI-2 variants produced can then be screened by the techniques described in Examples 1 and 2 to determine whether particular variants lack the protease sensitivity of PAI-2 in the 66-98 amino acid residue region as evidenced by the absence of the 37kD form and tested for maintenance of biological activity of PAI-2 as described in Examples 1 and 2 for Δ 66-98 and Δ 74-96.

The compositions of the invention are prepared by mixing, preferably homogeneously mixing, variant with a pharmaceutically acceptable carrier, diluent, and/or excipient using standard methods of pharmaceutical preparation.

The amount of variant required to produce a single dosage form will vary depending upon the condition to be treated, host to be treated and the particular mode of administration. The specific dose level for any particular patient will depend upon a variety of factors including the activity of the variant employed, the age, body weight, general health, sex, and diet of the patient, time of administration, route of administration, rate of excretion, drug combination and the severity of the condition undergoing treatment. The amounts required may be determined in accordance with standard pharmaceutical techniques.

The composition may be administered parenterally in unit dosage formulations containing conventional, non-toxic, pharmaceutically acceptable carriers, diluents and/or excipients as desired.

Injectable preparations of the variants of the invention, for example, sterile injectable aqueous or oleaginous suspensions may be formulated according to the known arts using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

It is anticipated that it may be possible to deliver the variants of the invention orally or topically as appropriate delivery systems are developed.

Antibodies are raised using standard vaccination regimes in appropriate hosts. The host is vaccinated with a variant or composition of the invention. The

compositions used for vaccination purposes may include an adjuvant.

Suitable adjuvants for the vaccination of animals include but are not limited to oil emulsions such as Marcol 52: Montanide 888 (Marcol is a Trademark of Esso. Montanide is a Trademark of SEPPIC, Paris), squalane or squalene, Adjuvant 65 (containing peanut oil, mannide monooleate and aluminum monostearate), mineral gels such as aluminum hydroxide, aluminum phosphate, calcium phosphate and alum, surfactants such as hexadecylamine, octadecylamine, lysolecithin, dimethyldioctadecylammonium bromide, N,N-dioctadecyl -N',N'-bis(2-hydroxyethyl)-propanediamine, methoxyhexadecylglycerol and pluronic polyols, polyanions such as pyran, dextran sulfate, polyacrylic acid and carbopol, peptides and amino acids such as muramyl dipeptide, dimethylglycine, tuftsin and trehalose dimycolate. The variants of the present invention can also be administered following incorporation into liposomes or other micro-carriers, or after conjugation to polysaccharides, proteins or polymers. Other adjuvants suitable for use in the present invention include conjugates comprising the variant together with an integral membrane protein of prokaryotic or eukaryotic origin, such as TraT.

Routes of administration, dosages to be administered as well as frequency of injections are all factors which can be optimized using ordinary skill in the art. Typically, the initial vaccination is followed some weeks later by one or more "booster" vaccinations, the net effect of which is the production of high titres of antibodies against the variant.

Monoclonal antibodies against the variants of the invention can be prepared using standard techniques for monoclonal antibody production.

The antibody composition is prepared by mixing, preferably homogeneously mixing, antibody with a pharmaceutically acceptable carrier, diluent and/or excipient using standard methods of pharmaceutical preparation.

Conjugates are prepared using standard techniques for conjugate synthesis. The conjugate may be prepared chemically using linking agents as necessary or by recombinant DNA techniques to provide a PAI-2 variant of the invention linked to a cytotoxic drug.

The conjugate composition is prepared by mixing, preferably homogeneously mixing, conjugate with a pharmaceutically acceptable carrier, diluent and/or excipient using standard methods of pharmaceutical preparation.

The amount of conjugate required to produce a single dosage form will vary depending upon the condition to be treated, host to be treated and the particular mode of administration. The specific dose level for any particular patient will depend upon a variety of factors including the activity of the conjugate employed, the age, body weight, general health, sex, and diet of the patient, time of administration, route of administration, rate of excretion, drug combination and the severity of the condition undergoing treatment. The amounts to be used can be determined by standard pharmaceutical techniques.

The conjugate composition may be administered parenterally, in unit dosage formulations containing conventional, non-toxic, pharmaceutically acceptable carriers, diluents, and/or excipients as desired.

Diagnostic kits are prepared by formulating antibodies at appropriate concentration with a pharmaceutically acceptable carrier, diluent, and/or excipient. A positive control standard of a known concentration of a variant of the invention is prepared similarly. The negative standard comprises carrier, diluent, and/or excipient alone. Examples of diagnostic kits include a tumour diagnostic wherein the reagent comprises an antibody of the invention and the positive control comprises a variant of the invention.

PLASMIDS

Various plasmids used in this work were derived from pBTA 438. Plasmid pBTA 438 consists of a 1.6kb cDNA

encoding PAI-2 cloned in pUC18 [Yanisch-Perron et al, Gene 33: 103-119 (1985)]. pBTA 438 was used to transform E. coli strain JM109 [Yanisch-Perron et al, Gene 33:103-119 (1985)] to yield strain BTA 1445, which was deposited with the American Type Culture Collection of 12301 Parklawn Drive, Rockville, MD 20852, USA under accession number ATCC 53585 on February 11 1987.

Plasmid pBTA641 can be derived from pBTA438 as follows. pBTA 438 is partially digested with XhoII plus DraI and a 1550bp fragment isolated and ligated to vector pLK58 cut with BglII and SmaI. The resultant plasmid pBTA 446 was linearized with BglII and ligated to a synthetic double stranded 27 mer oligonucleotide having the sequence $GATCT(N)_{16}ATGGAG$ (SEQ ID NO. 12), wherein N represents any nucleotide, containing a bacterial ribosome binding site and the initial nucleotides of the native PAI-2 gene, creating plasmid pBTA641. Plasmid pBTA447 is identical to pBTA 641 except that a 26 mer oligonucleotide containing a bacterial ribosome binding site having the sequence $GATCT(N)_{15}ATGGAG$ (SEQ ID NO. 13) was used instead of the 27 mer.

Plasmid pMINS71 was derived as follows: the BglII-EcoR1 PAI-2 gene fragment from pBTA 641 was inserted into pSp72 (Promega) at the BglII/EcoR1 sites; the BglII-SacI PAI-2 gene fragment from this vector was inserted into the HindIII/SacI sites of pGEM4Z (Promega) in a three way ligation with a synthetic adaptor with cohesive HindIII-BglII ends to create pMINS71.

PREPARATIVE EXAMPLE 1

A. Bacterial Expression of PAI-2

Cell extracts of induced (by incubating cells at 42°C) and uninduced (incubated at 30°C) E. coli K-12 host cells containing pBTA447 and pBTA641 were screened for the presence of PAI-2 using affinity purified monoclonal (Biopool) or polyclonal antibodies to human PAI-2. Biological activity was assessed by a shift in the electrophoretic mobility in the presence of urokinase, characteristic of the formation of a urokinase-PAI-2

complex. As shown in Fig. 3 a PAI-2 protein band (M_r 46kD), visualized by western transfer using a monoclonal antibody to human placental inhibitor and iodinated protein A, is present in the induced (42°C) samples. A lower molecular weight (M_r 37,000) immunologically cross-reactive protein band was also observed indicating possible proteolytic cleavage of the PAI-2 molecule.

B. Purification of 37kD Form

(i) Cell Growth and Lysis

E. coli K-12 cells harbouring the plasmid pBTA447 were heat induced at 38°C in a 10L fermenter for 24h and the cells then recovered by centrifuging at 17,000 xg for 20 min. A total of 524g wet weight of cells was recovered from 8L of fermentation broth.

The cells (524g) were suspended in 1500ml of 0.1M Na phosphate buffer, pH7.0 containing 1mM EDTA and 1mM PMSF at 4°C and lysed by four passages through a Martin-Gaulin press at 8000 psi. The press was washed out with 300ml of the above buffer and the lysate and washes combined. To this solution was added $MgCl_2$ to a final concentration of 2mM and the solution centrifuged at 17,700 xg for 60 mins. The supernatant (1600ml) resulting from this centrifugation was recentrifuged at 30,100 xg for 60 mins to remove remaining insoluble material and the supernatant recovered. To this supernatant (1570 ml) was added 574.6g of solid ammonium sulphate to give a 60% saturated solution, the solution stirred for 15 min. and then centrifuged at 30,000xg for 30 mins. The resultant pellet, which contains the PAI-2 was divided into eighths and stored at -20°C.

(ii) DEAE-Sephacel Chromatography

One eighth aliquot of 0-60% ammonium sulphate precipitate was dissolved in 200ml of 0.1M Na phosphate, pH7.0 containing 1mM EDTA and 0.1M DTT, and incubated at 37°C for 90 min. This solution was then diluted to 500ml with 0.1M Na phosphate, pH 7.0 containing 1mM EDTA and 0.05% 2-mercaptoethanol and dialysed at 4°C against the same buffer for 48h. The dialysed solution was then

applied to a DEAE-Sephacel column (4.4 cm x 10 cm) equilibrated in the above buffer and eluted until the absorbance at 280nm returned to base line. A linear 2 litre gradient from 0 to 0.5M NaCl in the same buffer was applied and the column eluted at a flow rate of 2ml min^{-1}. Fractions of 10ml were collected and 200µl aliquots analysed by SDS-PAGE and western analysis. The PAI-2 eluted between 58mM and 81mM NaCl under these conditions and these fractions were pooled and dialysed against 1mM Na phosphate, pH 7.0 containing 0.05% 2-mercaptoethanol for 48h at 4°C.

(iii) Hydroxylapatite Chromatography

The dialysed PAI-2 from the previous step was applied to a 3.2cm x 15cm column of Biogel HPT equilibrated in 1mM Na phosphate, pH7.0 containing 0.05% 2-mercaptoethanol and the column washed with the same buffer until the absorbance at 280nm had returned to baseline. A one litre linear gradient from 1mM Na phosphate to 200mM Na phosphate was then applied and the column eluted at a flow rate of 1ml min^{-1}. Six ml fractions were collected. The PAI-2 eluting from the column was detected using SDS-PAGE and western blotting and revealed under reducing conditions two distinct immunologically cross-reactive protein bands. The molecular weights of these two forms of PAI-2 were ca. 46kD and ca. 37kD.

(iv) High Pressure Liquid Chromatography

To resolve these two forms of PAI-2 an aliquot from the Biogel HPT column containing PAI-2 was chromatographed on a Vydac C4 HPLC column using a gradient of acetonitrile in 0.1% TFA. This chromatograph revealed two major peaks, the former containing the 37kD form of PAI-2 and the latter containing the 46kD form of PAI-2, as determined by non-reducing SDS-PAGE. Amino acid sequencing of the ca. 37kD form of PAI-2 revealed the sequence - Lys Gly Ser Tyr Pro Asp Ala Ile Leu Gln Ala Gln Ala Ala Asp (SEQ ID NO. 14).

This sequence corresponds to a form of PAI-2 starting at residue 87 of the mature form of PAI-2 and

suggests that the glutamine 86-lysine 87 (Q86-K87) peptide bond is highly susceptible to proteolysis. A similar immunologically cross reactive form of PAI-2 of ca. 37kD was also observed during the purification of naturally occurring PAI-2 from U937 cells suggesting that proteolytic cleavage at the Q86-K87 peptide bond occurs in both mammalian and bacterial cells and supporting the concept that this bond is highly labile.

C. Purification of 37kD Form

E. coli cells harbouring the plasmid pBTA641 were heat induced at 38°C in a 10 litre fermenter for 24 hours and the cells then recovered by centrifugation at 17,000 xg for 20 mins.

(i) Cell Lysis

The cell pellet obtained from 5 litres of this fermentation was suspended in 800ml of 50mM Na phosphate, containing 1 mM EDTA, 10mM ε-amino caproic acid (ε-ACA) and 10mM 2-mercaptoethanol, pH 6.6, and lysed by six passages through a Martin-Gaulin 15 MR homogenizer at 9000 psi. To the resultant lysate (900ml) was added $MgCl_2$ to 2mM and the suspension centrifuged at 17,700xg for 1 hour at 4°C.

(ii) Ammonium Sulphate Precipitation

To the supernatant from the above centrifugation was added solid ammonium sulphate to give a 30% saturated solution. The solution was stirred for 30 mins at 4°C and then the precipitate removed by centrifugation at 17,700xg for 1 hour at 4°C. The supernatant (760ml) was adjusted to 50% saturation by the addition of more solid ammonium sulphate and following stirring at 4°C for 30 min, the suspension was centrifuged at 17,700xg for 1 hour at 4°C. The pellet recovered from this precipitation step was dissolved in Buffer B (50mM Na citrate, 1mM EDTA, 10mM ε-ACA and 10mM 2-mercaptoethanol, pH 5.5) to give a final volume of 200ml. This solution was then dialysed against 20 volumes of Buffer B overnight at 4°C.

(iii) Phenyl Sepharose Chromatography

The dialysed solution was made 1M in ammonium sulphate and the sample (286ml) was applied to a Phenyl Sepharose column (5cm x 19cm; Vt = 373ml), equilibrated in Buffer A, (Buffer A is Buffer B containing 1M ammonium sulphate) at a flow rate of 100ml/h. Following loading of the sample the column was washed with Buffer A until the absorbance at 280nm (A280) returned to baseline and then a linear gradient of 800ml of Buffer A and 800ml of Buffer B applied. Fractions of 10ml were collected. Following completion of the gradient the column was washed with 50mM glycine, pH9.0 until the A_{280} returned to baseline. The PAI-2 eluted in fraction 75-150 as determined by the urokinase inhibition assay of Coleman and Green (in Methods in Enzymology 80: 408-414 1981) and by an immunological dot blot assay. These fractions were pooled (850ml) and precipitated by the addition of ammonium sulphate to 60% saturation. The pellet was recovered by centrifigation at 17,700xg for 30 mins at 4°C and dissolved in Buffer C (25mM Na borate, 1mM EDTA, 10mM ε-ACA, 10mM 2-mercaptoethanol, pH9.0).

(iv) Sephacryl S200 Chromatography

The solution containing PAI-2 (25ml) was applied to a Sephacryl S200 column (3.3 cm x 95 cm) equilibrated in Buffer C and eluted at a flow rate of 40ml/h. Fractions of 6ml were collected and analysed for PAI-2 by urokinase inhibition, SDS-PAGE and immunological cross-reactivity in a dot blot assay. Fractions containing the PAI-2 (fractions 52-90) were pooled and precipitated with 60% saturated ammonium sulphate. The pellet was recovered by centrifugation at 17,700xg for 30 mins at 4°C and redissolved in Buffer A. The pH of this solution was adjusted to 5.5 with HCl and the precipitate which developed was removed by centrifugation at 17,700xg for 30 mins at 4°C.

(v) Second Phenyl Sepharose Chromatography

The supernatant was applied to a Phenyl Sepharose column (5cm x 10cm) at a flow rate of 60ml/h. Following loading, the column was washed with Buffer A and then a

linear gradient of Buffer A and Buffer B applied as described in (iii) above. Following completion of the gradient the column was washed with 50mM glycine, pH9 and the fractions containing PAI-2 identified by SDS-PAGE and western blotting. Fractions 15-27, containing the PAI-2, were pooled, precipitated with 60% ammonium sulphate and redissolved in 15ml of Buffer C.

(vi) Second Sephacryl S 200 Chromatography

The sample containing PAI-2 from the second Phenyl Sepharose column above was applied to a Sephacryl S200 column (2.5cm x 95cm) equilibrated in Buffer C and eluted at a flow rate of 30ml/h. Fractions of 2.6ml were collected and analysed for PAI-2 by SDS-PAGE.

(viii) Reverse Phase HPLC

The SDS-PAGE of the fractions from the second Sephacryl S200 column showed the presence of two proteins with approximate molecular weights of ca 46kD and 37kD when electrophoresed in the presence of 2-mercaptoethanol. These protein bands are similar to those observed in "B. Purification of 37kD Form ". To resolve these two forms, a 90µl aliquot of fraction 106 from the second Sephacryl S-200 column above was chromatographed on a Vydac C_4 reverse phase HPLC column using a gradient of acetonitrile in 0.1% TFA. The leading edge of the major absorbance peak eluted from this column contained primarily the 37kD protein. Amino acid sequencing of this fraction revealed an N-Terminal sequence of F M Q Q I Q K G S Y (Phe Met Gln Gln Ile Gln Lys Gly Ser Tyr: SEQ ID NO. 15) which corresponds to the sequence of PAI-2 starting at amino acid residue 81. It was therefore concluded that this form of PAI-2 arose from proteolytic cleavage of the mature form of PAI-2 at the glycine 80-phenylalanine 81 bond.

The observation that purification of PAI-2 overexpressed in E. coli by this alternative method and, in particular, the inclusion of ε-ACA as an inhibitor of lysine specific proteases, protected PAI-2 from cleavage at the Q86-K87 bond but not cleavage at a region only six amino acids upstream of this site, reinforces the view that

this region of the molecule is highly susceptible to protease cleavage.

D. Purification of 37kD Form

To determine whether the proteolysis observed above could be prevented by expression in an alternative host PAI-2 was overexpressed in the baculovirus insect cell system (Lucknow and Summers, Biotechnology 6: 47-55, 1988). The expressed product was purified essentially as described in "C. Purification of 37kD Form" using steps (i) through (iv) except that the 50% ammonium sulphate precipitation step in (ii) was omitted. The PAI-2 eluting from the Sephacryl S-200 column was detected by SDS-PAGE and western blotting under reducing conditions. This analysis showed the presence of both a 46kD and a 37kD form of PAI-2, indicating that proteolytic cleavage of the molecule was occurring as observed previously. To further define the site of this cleavage the PAI-2 pool obtained from the Sephacryl S-200 chromatography step was dialysed against 20mM glycine, 10mM EDTA and 10mM 2-mercaptoethanol, pH9.0 and the sample (30ml) then applied to a Q-Sepharose column (0.9cm x 24cm) at a flow rate of 60ml/h. The PAI-2 eluted unretarded from this column and on SDS-PAGE revealed two Coomassie blue staining bands of Mr=ca 37kD and ca 46kD. N-terminal amino acid sequencing of an aliquot of this material revealed a single sequence as shown below G F M Q Q I Q K G S Y P D A I (i.e. Gly Phe Met Gln Gln Ile Gln Lys Gly Ser Tyr Pro Asp Ala Ile : SEQ ID NO. 16). No authentic N-terminal sequence for the full length PAI-2 was observed, indicating that the 46kD form of PAI-2 when expressed in insect cells contains a blocked N-terminus. Similar results have been observed with full length PAI-2 isolated from U937 cells (Kruithof et al J. Biol Chem 216: 11207-11213 1986) and from placenta (Andreasen et al 261: 7644-7651 1986). The observed sequence is consistent with proteolytic cleavage occurring between cysteine 79 and glycine 80, only one peptide bond upstream from the G80-F81 cleavage site observed with PAI-2 purified from E. coli in "C. Purification of 37kD Form".

These results further confirm the high degree of proteolytic susceptibility of this region of the PAI-2 molecule.

E. Purification of the K87A variant of PAI-2

Creation of a variant PAI-2, wherein amino acid residue 87 was changed from Lys to Ala, was achieved by site-directed mutagenesis, after transferring the PAI-2 coding region to the phage M13 vector mp18. Preparation and use of single-stranded phage DNA, as well as the use of the two oligonucleotides containing the mutated sequence [(5'-CAG CAG ATC CAG GCA GGT AGT TAT CCT-3' (SEQ ID NO. 17), 5'-AGG ATA ACT ACC TGC CTG GAT CTG CTG-3' complement of SEQ ID NO. 17)], were carried out as previously described (Amersham; oligonucleotide-directed in vitro mutagenesis system).

The K87A variant of PAI-2 was purified from a 1 litre culture of E. coli K-12 cells harbouring the plasmid pBTA674. This plasmid is identical to pBTA641 but with the PAI-2 DNA replaced with the variant form of PAI-2.

The purification was performed essentially as described in "C. Purification of 37kD Form", steps (i) through (iv) except that the 50% ammonium sulphate precipitation in step (ii) was omitted. Analysis of the fractions eluted from the Sephacryl S-200 column by reducing SDS-PAGE and western blotting indicated the presence of both a 46kD and a 37kD form of PAI-2, indicating that mutagenesis of lysine 87 to an alanine residue failed to prevent cleavage of the PAI-2 molecule in the region previously identified as protease sensitive (see B. Purification of 37kD form).

Example 1

Deletion of Protease Sensitive Site

The HinfI-PstI segment spanning the protease sensitive site in PAI-2 was replaced by synthetic oligonucleotides with cohesive HinfI and PstI ends (see Fig. 5), creating a variant PAI-2 in which amino acids 74 to 96 inclusive were deleted. The events involved in the construction of this deletion variant are illustrated in

Fig. 6. In essence, the deletion variant was assembled in an intermediate vector by a three way ligation between a BglII - HinfI fragment from pBTA641, a PstI-BglII fragment from pMINS71 and the annealed oligonucleotides A1 (SEQ ID NO. 7) and A2 (SEQ ID NO. 8). The assembled deletion PAI-2 was then excised from the intermediate vector and exchanged with native PAI-2 in pBTA641 to create pBTA 829.

Oligonucleotides (Figure 5 SEQ ID NOs. 7 and 8) were synthesized on an Applied Biosystems DNA synthesizer (Model 380A), and purified through a polyacrylamide gel. Complementary oligonucleotides (A1: SEQ ID NO. 7 and A2 SEQ ID NO. 8) were mixed in a 1:1 molar ratio and phosphorylated using 5 units T4 polynucleotide kinase in 65mM Tris-Cl pH 7.5, 10mM $MgCl_2$, 5mM dithiothreitol, 1mM ATP. The mixture was heated to 65°C for 10 minutes and cooled slowly to room temperature to allow annealing to take place. The various restriction fragments were prepared as follows. Restriction enzyme digests of purified plasmid DNA were carried out in buffers recommended by the supplier. Required DNA fragments were separated from the plasmid by gel electrophoresis through 0.8-1.5% Sea-Plaque agarose (FMC Corporation) in Tris-acetate buffer (Maniatis et al, 1982). Fragments were visualized by staining with ethidium bromide and UV transillumination. The band of agarose containing the appropriate fragment was sliced out of the gel, melted at 65°C and the DNA was extracted three times with phenol/chloroform/isoamyl alcohol. The DNA was then precipitated with ethanol.

Vectors were typically prepared as follows. Plasmid DNA was digested with the appropriate restriction enzymes, the digest was extracted with an equal volume of phenol/chloroform/isoamyl alcohol and the DNA precipitated with 2.5 volumes of ethanol. The digested DNA was resuspended in 50mM Tris-Cl pH 9.0, 1mM $MgCl_2$, 0.1mM $ZnCl_2$, 1mM spermidine and incubated with 1-2 units calf intestinal alkaline phosphatase (Boehringer Mannheim) for 30-60 mins at 37°C. The enzyme was heat killed at 70°C

for 15 minutes then the DNA was extracted with phenol/chloroform/isoamyl alcohol and precipitated with ethanol.

Ligations were carried out as follows. Vector and insert DNAs were mixed at a molar ratio of between 1:1 and 1:5 (1:10 if the insert was smaller then 100bp) in 1mM ATP, 10mM $MgCl_2$, 5mM DTT, 65mM Tris-Cl pH7.5 in a volume of 20µl. Ligations were carried out at 16°C overnight with 0.5-1 unit T4 DNA ligase (Boehringer Mannheim). From ligation mixes 5-10µl was removed for transformation into a competent E. coli K12 host (Hanahan, J. Mol Biol 166: 557-580, 1983). Transformants were selected by plating onto tryptone-soya agar plates containing 100µg/ml ampicillin.

Plasmid DNA was extracted from individual colonies and the correct recombinant plasmids identified by restriction analysis. The region of the PAI-2 gene where the deletion was made was sequenced to confirm the changes. Sequencing was carried out on double-stranded plasmid DNA using the Sequenase DNA Sequencing Kit (USB) as described in the instruction manual. The primer used was the T7 primer (Promega).

Bacterial Constructions and Expression

The complete coding sequence of PAI-2 and the deletion variant were placed under the control of the lambda P_L promoter in the vector pLK58, with a synthetic oligonucleotide upstream of the ATG providing a bacterial ribosome binding site at an appropriate distance from the start codon, giving plasmids pBTA 641 encoding the native PAI-2 sequence and pBTA829 encoding the deletion variant Δ74-96.

These plasmids were used to transform an E. coli K-12 ΔHI Δtrp host which contained the thermolabile repressor of lambda, cI857. Transformed cells were grown overnight in TSB medium (Oxoid) at 28°C. Cells were then diluted in MEB medium (Mott et al Proc. Natl. Acad. Sci. 82: 88-92 1985), grown at 28°C to an OD_{600} of 1.0 when prewarmed (48°C) MEB medium was added in equal volume to

equilibrate the temperature to 38°C. Following 4 hours of growth at 38°C the cells were harvested by centrifugation at 8000 x g for 15 mins. Cell pellets were resuspended in Behs buffer (10mM p-chloromercuro benzoic acid, 10mM EDTA $(Na)_2$, 10mM 1,10-phenathioline, 100mM phosphate, pH7.0) and lysed by two passes through a french press at 16,000 psi (on ice). The supernatant from lysed cells was clarified by centrifugation at 8000 xg for 15 mins and tested for the presence of PAI-2 using affinity purified monoclonal (Biopool) or polyclonal antibodies to human PAI-2. Biological activity was also assessed by a shift in the electrophoretic mobility in the presence of urokinase, characteristic of the formation of a urokinase - PAI-2 complex, as described below.

U-PA Binding Experiment

The ability of the deletion variant of PAI-2 described above to bind to urokinase was determined in a urokinase binding experiment. Since the Δ74-96 variant is a significantly altered molecule compared to the native PAI-2 it is not possible to predict whether the variant has biological activity or not. Urokinase (LMW, American Diagnostica) was added to clarified supernatant from lysed cells expressing native (i.e. expressed from pBTA641) or variant Δ74-96 (i.e. expressed from pBTA829) PAI-2, or no PAI-2 (i.e. cells containing pBTA 836). As a negative control, lysates were used without the addition of urokinase. Plasmid pBTA 836 was derived from pBTA 641 by digestion with BglII and EcoRl to excise the PAI-2 gene, followed by a fill-in reaction using Klenow enzyme and a ligation reaction to reform an intact plasmid lacking the PAI-2 gene.

Binding was allowed to proceed at room temperature for 90 minutes. Samples were then boiled for 3 minutes after the addition of a buffer containing SDS and 2-mercaptoethanol and analysed by SDS-PAGE and western blotting, using either a goat polyclonal antibody against PAI-2 (Fig. 7A), or rabbit polyclonal antibody against urokinase (Fig. 7B). Bound antibody was detected using a

second antibody-HRP conjugate directed against the primary antibody.

Figure 7 shows the results of such an experiment. The addition of urokinase to lysates containing native or variant Δ74-96 PAI-2 resulted in the formation of an SDS stable complex of approximately 69kD that reacted with either polyclonal antibody directed against PAI-2 (Fig. 7A, lanes c and e) or antibody directed against urokinase (Fig. 7B, lanes c and e). In the absence of urokinase, or in cell lysates lacking PAI-2, such a complex could not be detected using either antibody against PAI-2 (Fig. 7A, lanes b, d, f, g) or antibody against urokinase (Fig. 7B, lanes b, d, f, g). These results are characteristic of the formation of a urokinase-PAI-2 complex and indicate that both the native PAI-2 and the variant Δ74-96 PAI-2 are capable of binding urokinase and hence possess biochemical activity.

Elimination of Proteolytic Sensitivity

In E. coli cells expressing native PAI-2 (i.e. from pBTA 641) the major products detected by PAI-2 specific antibody following SDS-PAGE and western transfer are a 46kD form, representing native PAI-2, and a 37kD form representing a degradation product (Fig. 3, lanes 3 and 4; Fig. 7A, lane b). In cells expressing the variant Δ74-96 PAI-2 (i.e. from pBTA 829) the 37kD degradation product cannot be detected (Fig. 7A, lane d). These results show that the variant Δ74-96 PAI-2 does not possess the proteolytic sensitivity of the native PAI-2.

Example 2

Deletion of Protease Sensitive Site.

DNA sequences encoding amino acids 66-98 inclusive were deleted from the PAI-2 coding region using the polymerase chain reaction (PCR) technique of site-directed mutagenesis by overlap extension (Ho et al. Gene 77: 51-59, 1989). The oligonucleotides used in the PCR reactions are shown in Fig. 8 (SEQ ID NOs. 9, 10 and 11) and an outline

of the construction of this deletion variant illustrated in Fig. 9.

In brief, the PAI-2 DNA was transferred to an intermediate vector which was used in PCR reactions to generate a Bgl II/PstI fragment in which the sequences encoding amino acids 66-98 inclusive had been deleted. The PCR generated Bgl II/Pst I deletion fragment was exchanged for the native Bgl II/Pst I fragment in the intermediate vector and the entire Bgl II/Pst I region from five independent transformants sequenced. From one of these transformants, in which the only differences from the native PAI-2 was the deletion of sequences encoding amino acids 66-98 inclusive, the PAI-2 DNA was recovered and ligated into the vector pLK 58 to create pBTA 840.

Oligonucleotides (Fig. 8: SEQ ID NOs 9, 10 and 11) were synthesized on an Applied Biosystems DNA synthesizer (Model 380A), with the trityl group left on, and purified on oligonucleotide purification cartridges (Applied Biosystems, Cat. No. 400771) according to the manufacturer's instructions. One oligo, Sp6 primer, was purchased from Promega.

PCR reactions were in 50mM KCl, 10mM tris-HCl pH 8.3, 1.5mM Mg Cl_2, 0.01% gelatin w/v, 200µM dNTPs, 2.5U amplitaq (Perkin-Elmer Cetus), using 100 pmoles of oligonucleotides and 0.35 pmoles of Eco R1 linearized PAI-2 plasmid. Reactions were carried out on a Gene Machine (Innovonics) set for 25 cycles with 1 minute denaturation (94°C), 1 minute annealing (50°C) and 1 minute extension (74°C). PCR products were separated from oligonucleotides either on Sephacryl S-200 columns or by gel electrophoresis through 1.5% sea-plaque agarose (FMC Corporation) in tris-acetate buffer (Maniatis et al 1982), followed by staining with ethidium bromide, visualization on a UV transilluminator and purification from the agarose gel slice on NACS columns (BRL) according to the manufacturer's instructions.

Other required DNA fragments were separated from plasmid DNA by gel electrophoresis through 0.8-1.5%,

sea-plaque agarose and purified as described above for PCR products.

Vectors were typically prepared as follows. Plasmid DNA was digested with the appropriate restriction enzymes for 1 to 2 hrs. at 37°C. Calf intestinal alkaline phosphatase (CIAP Boehringer Mannheim, 1 to 2 units) was added directly to the restriction digest and the incubation continued at 37°C for 1 hour. In some cases, when restriction enzymes that yielded flush or 3' overhang ends were used, the incubation with CIAP was for 30 minutes at 37°C and 30 minutes at 50°C. The CIAP enzyme was heat killed at 70°C for 15 minutes and the DNA was extracted with phenol/chloroform/isoamyl alcohol and precipitated with ethanol.

Ligations and transformation of E.coli K12 hosts were as described in Example 1. In some cases ligations were at 4°C for 48 hours or 16°C for 4 to 6 hours.

Sequencing of the Bgl II/Pst I regions were performed on double-stranded plasmid DNA, after alkali denaturation, using a Multiwell Microtitre Sequencing System Kit (Amersham) as described in the instruction manual. The primer used was the Sp6 primer (Promega).

Plasmids used in this work were derived as described above.

Expression in E. coli.

Plasmid pBTA 840 was used to transform an E. coli $\Delta H_1 \Delta$trp host which contained the thermolabile repressor of lambda, cI857. A single transformant was grown overnight in TSB medium at 28°C and the resulting culture used to innoculate a 10 litre fermenter. The E. coli cells were heat induced at 38°C for 24 hrs. and the cells recovered by centrifugation at 17,000g for 20 min.

Purification of Δ66-98 and Δ74-96 PAI-2

The PAI-2 variants Δ66-98 and Δ74-96 can be purified from cells of E. coli expressing the molecule using a combination of the procedures used in the purification of the native molecule viz processes involving phenyl-sepharose chromatography, Sephacryl S200

chromatography, ion exchange chromatography and/or reverse phase HPLC. These procedures are described in International Patent Application No PCT/AU85/00191 (WO 86/01212) and International Patent Application No PCT/AU87/00068 (WO 87/05628) and the results of purifying Δ66-98 are illustrated in Figure 10.

U-PA, two chain t-PA, and single chain t-PA binding experiment.

The ability of the purified deletion variant Δ66-98 to bind to U-PA, to two chain t-PA and to single chain t-PA was examined in a binding experiment similar to that described in Example 1. The binding characteristics of Δ66-98 PAI-2 (SEQ ID NO. 3) were compared to those exhibited by native PAI-2 (i.e. as expressed from pBTA641) (SEQ ID NO. 1), the Δ74-96 variant (i.e. as expressed from pBTA829) (SEQ ID NO 2) and to the second form of native PAI-2 that differs by three amino acids from the PAI-2 expressed from pBTA641 (Schleuning et. al. Mol. Cell. Biol. 7: 4564-4567, 1987). The alternative native PAI-2 was expressed from pBTA 683. Plasmid pBTA 683 was derived from pBTA641 by site directed mutagenesis that changed 3 amino acids to that found in the second form of PAI-2. [Schleuning et al. Mol. Cell Biol. 7: 4564-4567 (1987)].

The various PAI-2s (0.25µg each) were incubated with either u-PA (3.75µg, Behring), two chain t-PA or single chain t-PA (3.75µg each, American Diagnostica) at room temperature for 160 minutes in 25mM Tris-HCl pH7.5, 75mM NaCl, 2.5mM EDTA and 0.5% TX-100. Samples were analysed on 10% SDS-polyacrylamide gels followed by western blotting. Blots were probed with a goat polyclonal antibody against PAI-2 and bound antibody detected by an anti-goat-HRP conjugate (Fig. 11).

All PAI-2s, (the two native forms and the two deletion variants) displayed identical binding characteristics. Thus, on incubation with either U-PA, two chain t-PA or single chain t-PA high molecular weight SDS stable forms of PAI-2 were seen. Such high molecular weight forms are characteristic of the formation of complexes between PAI-2 and these plasminogen activators.

Elimination of Proteolytic Sensitivity

As with the variant Δ74-96, the 37kD degradation product observed on purification of native PAI-2, was not found in purified preparations of the variant Δ66-98 (Fig. 10).

INDUSTRIAL APPLICABILITY

The PAI-2 variants of the invention can be used as therapeutic and diagnostic agents in patients with tumours, or suffering from chronic inflammatory conditions such as rheumatoid arthritis.

Other conditions where the application of a specific PA inhibitor may be of use include diseases or conditions such as osteoarthritis, multiple sclerosis, colitis ulcerosa, SLE-like disease, psoriasis, pemphigus, corneal ulcer, gastroduodenal ulcer, purpura, periodontitis, haemorrhage and muscular dystrophy. A specific PA inhibitor would also be useful as an adjunct to thrombolytic therapy involving PAs in order to reduce the incidence and severity of the side effect of such treatment viz. systemic fibrinolysis. Finally, a PA inhibitor could have a significant role in skin wound healing and tissue repair especially since two trypsin inhibitors have been shown to enhance formation of connective tissue with increased tensile strength of the wound tissue [Kwaan, HC and Astrup, T (1969) Exp. Molec. Path 11, 82] and keratinocytes are known to produce both uPA and tPA [Grondahl-Hansen, J et al. (1988) J. Invest Dermatol.].

Antibodies against variants of the invention should be useful in the detection or monitoring of disease states or conditions such as monocytic leukaemia, cancer, foetal development and chronic inflammatory diseases.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANTS: Goss, Neil Howard (for US)
Richardson, Michael Andrew (for US)
Biotech Australia Pty Limited (for designated states other than the USA)

(ii) TITLE OF INVENTION: VARIANTS OF PAI-2

(iii) NUMBER OF SEQUENCES: 18

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Griffith Hack & Co
(B) STREET: 71 York Street
(C) CITY: Sydney
(D) STATE: New South Wales
(E) COUNTRY: AUSTRALIA
(F) ZIP: 2000

(v) COMPUTER READABLE FORM

(A) MEDIUM TYPE: 3 1/2 inch 2DD floppy disc
(B) COMPUTER: Macintosh
(C) OPERATING SYSTEM: Macintosh, 6.04 and above
(D) SOFTWARE: Microsoft word 4

(vi) CURRENT APPLICATION DATA: Not available
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: AU PJ7924
(B) FILING DATE: 20 December 1989

(2) INFORMATION FOR SEQ ID NO. I

(i)	SEQUENCE CHARACTERISTICS:	
(A)	LENGTH:	1610 base pairs
(B)	TYPE:	Nucleic acid
(C)	STRANDEDNESS:	Double stranded
(D)	TOPOLOGY:	Linear
(ii)	MOLECULE TYPE:	cDNA to mRNA
(A)	DESCRIPTION:	Codes for human plasminogen activator inhibitor type 2 protein
(iii)	HYPOTHETICAL:	No
(iv)	ANTI-SENSE:	No
(v)	FRAGMENT TYPE:	N/A
(vi)	ORIGINAL SOURCE:	
(A)	ORGANISM:	Homo sapiens
(B)	STRAIN:	
(C)	INDIVIDUAL ISOLATE:	
(D)	DEVELOPMENTAL STAGE:	
(E)	HAPLOTYPE:	
(F)	TISSUE TYPE:	
(G)	CELL TYPE:	Monocyte
(H)	CELL LINE:	U937
(I)	ORGANELLE:	
(vii)	IMMEDIATE SOURCE:	
(A)	LIBRARY:	
(B)	CLONE:	BTA 1445

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: 18

(B) MAP POSITION: 18q21-q23

(C) UNITS:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 1

GTCAGACAGC AACTCAGAGA ATAACCAGAG AACAACCAGA TTGAAACA 48

ATG GAG GAT CTT TGT GTG GCA AAC ACA CTC TTT GCC CTC AAT TTA 93
MET Glu Asp Leu Cys Val Ala Asn Thr Leu Phe Ala Leu Asn Leu
5 10 15

TTC AAG CAT CTG GCA AAA GCA AGC CCC ACC CAG AAC CTC TTC CTC 138
Phe Lys His Leu Ala Lys Ala Ser Pro Thr Gln Asn Leu Phe Leu
20 25 30

TCC CCA TGG AGC ATC TCG TCC ACC ATG GCC ATG GTC TAC ATG GGC 183
Ser Pro Trp Ser Ile Ser Ser Thr MET Ala MET Val Tyr MET Gly
35 40 45

TCC AGG GGC AGC ACC GAA GAC CAG ATG GCC AAG GTG CTT CAG TTT 228
Ser Arg Gly Ser Thr Glu Asp Gln MET Ala Lys Val Leu Gln Phe
50 55 60

AAT GAA GTG GGA GCC AAT GCA GTT ACC CCC ATG ACT CCA GAG AAC 273
Asn Glu Val Gly Ala Asn Ala Val Thr Pro MET Thr Pro Glu Asn
65 70 75

TTT ACC AGC TGT GGG TTC ATG CAG CAG ATC CAG AAG GGT AGT TAT 318
Phe Thr Ser Cys Gly Phe MET Gln Gln Ile Gln Lys Gly Ser Tyr
80 85 90

CCT GAT GCG ATT TTG CAG GCA CAA GCT GCA GAT AAA ATC CAT TCA 363
Pro Asp Ala Ile Leu Gln Ala Gln Ala Ala Asp Lys Ile His Ser
95 100 105

TCC TTC CGC TCT CTC AGC TCT GCA ATC AAT GCA TCC ACA GGG AAT 408
Ser Phe Arg Ser Leu Ser Ser Ala Ile Asn Ala Ser Thr Gly Asn
110 115 120

TAT TTA CTG GAA AGT GTC AAT AAG CTG TTT GGT GAG AAG TCT GCG 453
Tyr Leu Leu Glu Ser Val Asn Lys Leu Phe Gly Glu Lys Ser Ala
125 130 135

AGC TTC CGG GAA GAA TAT ATT CGA CTC TGT CAG AAA TAT TAC TCC 498
Ser Phe Arg Glu Glu Tyr Ile Arg Leu Cys Gln Lys Tyr Tyr Ser
140 145 150

TCA GAA CCC CAG GCA GTA GAC TTC CTA GAA TGT GCA GAA GAA GCT 543
Ser Glu Pro Gln Ala Val Asp Phe Leu Glu Cys Ala Glu Glu Ala
155 160 165

AGA AAA AAG ATT AAT TCC TGG GTC AAG ACT CAA ACC AAA GGC AAA 588
Arg Lys Lys Ile Asn Ser Trp Val Lys Thr Gln Thr Lys Gly Lys
170 175 180

ATC CCA AAC TTG TTA CCT GAA GGT TCT GTA GAT GGG GAT ACC AGG 633
Ile Pro Asn Leu Leu Pro Glu Gly Ser Val Asp Gly Asp Thr Arg
185 190 195

```
ATG GTC CTG GTG AAT GCT GTC TAC TTC AAA GGA AAG TGG AAA ACT  678
MET Val Leu Val Asn Ala Val Tyr Phe Lys Gly Lys Trp Lys Thr
                200                 205                 210

CCA TTT GAG AAG AAA CTA AAT GGC CTT TAT CCT TTC CGT GTA AAC  723
Pro Phe Glu Lys Lys Leu Asn Gly Leu Tyr Pro Phe Arg Val Asn
                215                 220                 225

TCG GCT CAG CGC ACA CCT GTA CAG ATG ATG TAC TTG CGT GAA AAG  768
Ser Ala Gln Arg Thr Pro Val Gln MET MET Tyr Leu Arg Glu Lys
                230                 235                 240

CTA AAC ATT GGA TAC ATA GAA GAC CTA AAG GCT CAG ATT CTA GAA  813
Leu Asn Ile Gly Tyr Ile Glu Asp Leu Lys Ala Gln Ile Leu Glu
                245                 250                 255

CTC CCA TAT GCT GGA GAT GTT AGC ATG TTC TTG TTG CTT CCA GAT  858
Leu Pro Tyr Ala Gly Asp Val Ser MET Phe Leu Leu Leu Pro Asp
                260                 265                 270

GAA ATT GCC GAT GTG TCC ACT GGC TTG GAG CTG CTG GAA AGT GAA  903
Glu Ile Ala Asp Val Ser Thr Gly Leu Glu Leu Leu Glu Ser Glu
                275                 280                 285

ATA ACC TAT GAC AAA CTC AAC AAG TGG ACC AGC AAA GAC AAA ATG  948
Ile Thr Tyr Asp Lys Leu Asn Lys Trp Thr Ser Lys Asp Lys MET
                290                 295                 300

GCT GAA GAT GAA GTT GAG GTA TAC ATA CCC CAG TTC AAA TTA GAA  993
Ala Glu Asp Glu Val Glu Val Tyr Ile Pro Gln Phe Lys Leu Glu
                305                 310                 315

GAG CAT TAT GAA CTC AGA TCC ATT CTG AGA AGC ATG GGC ATG GAG 1038
Glu His Tyr Glu Leu Arg Ser Ile Leu Arg Ser MET Gly MET Glu
                320                 325                 330

GAC GCC TTC AAC AAG GGA CGG GCC AAT TTC TCA GGG ATG TCG GAG 1083
Asp Ala Phe Asn Lys Gly Arg Ala Asn Phe Ser Gly MET Ser Glu
                335                 340                 345

AGG AAT GAC CTG TTT CTT TCT GAA GTG TTC CAC CAA GCC ATG GTG 1128
Arg Asn Asp Leu Phe Leu Ser Glu Val Phe His Gln Ala MET Val
                350                 355                 360

GAT GTG AAT GAG GAG GGC ACT GAA GCA GCC GCT GGC ACA GGA GGT 1173
Asp Val Asn Glu Glu Gly Thr Glu Ala Ala Ala Gly Thr Gly Gly
                365                 370                 375

GTT ATG ACA GGG AGA ACT GGA CAT GGA GGC CCA CAG TTT GTG GCA 1218
Val MET Thr Gly Arg Thr Gly His Gly Gly Pro Gln Phe Val Ala
                380                 385                 390
```

```
GAT CAT CCT TTT CTT TTT CTT ATT ATG CAT AAG ATA ACC AAC TGC 1263
Asp His Pro Phe Leu Phe Leu Ile MET His Lys Ile Thr Asn Cys
                395                 400                 405

ATT TTA TTT TTC GGC AGA TTT TCC TCA CCC TAAAACTAAG          1303
Ile Leu Phe Phe Gly Arg Phe Ser Ser PRO
                410                 415

CGTGCTGCTT CTGCAAAAGA TTTTTGTAGA TGAGCTGTGT GCCTCAGAAT      1353

TGCTATTTCA AATTGCCAAA AATTTAGAGA TGTTTTCTAC ATATTTCTGC      1403
TCTTCTGAAC AACTTCTGCT ACCCACTAAA TAAAAACACA GAAATAATTA      1453
GACAATTGTC TATTATAACA TGACAACCCT ATTAATCATT TGGTCTTCTA      1503
AAATGGGATC ATGCCCATTT AGATTTTCCT TACTATCAGT TTATTTTTAT      1553
AACATTAACT TTTACTTTGT TATTTATTAT TTTATATAAT GGTGAGTTTT      1603
TAAATTA                                                    1610
```

INFORMATION FOR SEQ ID NO. 2

(i)	SEQUENCE CHARACTERISTICS:	
(A)	LENGTH:	1512 base pairs
(B)	TYPE:	Nucleic acid
(C)	STRANDEDNESS:	Double stranded
(D)	TOPOLOGY:	Linear
(ii)	MOLECULE TYPE:	cDNA TO mRNA
(A)	DESCRIPTION:	Codes for human plasminogen activator inhibitor type 2 protein in which amino acids 74 to 96 inclusive have been deleted
(iii)	HYPOTHETICAL:	No
(iv)	ANTI-SENSE:	No
(v)	FRAGMENT TYPE:	N/A
(vi)	ORIGINAL SOURCE:	
(A)	ORGANISM:	Homo sapiens
(B)	STRAIN	
(C)	INDIVIDUAL ISOLATE:	
(D)	DEVELOPMENT STAGE:	
(E)	HAPLOTYPE:	
(F)	TISSUE TYPE:	
(G)	CELL TYPE:	Monocyte
(H)	CELL LINE:	U937
(I)	ORGANELLE:	

(vii)	IMMEDIATE SOURCE:	
(A)	LIBRARY:	
(B)	CLONE:	BTA 1916
(ix)	FEATURE:	
(A)	NAME/KEY	PAI-2 variant
(B)	LOCATION:	Amino acids 74-96
(C)	IDENTIFICATION METHOD:	By experiment
(D)	OTHER INFORMATION:	Removes a protease sensitive site, product binds to urokinase, tissue plasminogen activator

(ix) SEQUENCE DESCRIPTION: SEQ ID NO. 2

```
GATCTGTAAG GAGGTATATA A ATG GAG GAT CTT TGT GTG GCA                  42
                        Met Glu Asp Leu Cys Val Ala
                                    5

AAC ACA CTC TTT GCC CTC AAT TTA TTC AAG CAT CTG GCA                  81
Asn Thr Leu Phe Ala Leu Asn Leu Phe Lys His Leu Ala
          10                  15                  20

AAA GCA AGC CCC ACC CAG AAC CTC TTC CTC TCC CCA TGG                 120
Lys Ala Ser Pro Thr Gln Asn Leu Phe Leu Ser Pro Trp
                    25                  30

AGC ATC TCG TCC ACC ATG GCC ATG GTC TAC ATG GGC TCC                 159
Ser Ile Ser Ser Thr Met Ala Met Val Tyr Met Gly Ser
     35                  40                  45

AGG GGC AGC ACC GAA GAC CAG ATG GCC AAG GTG CTT CAG                 198
Arg Gly Ser Thr Glu Asp Gln Met Ala Lys Val Leu Gln
               50                  55

TTT AAT GAA GTG GGA GCC AAT GCA GTT ACC CCC ATG ACT                 237
Phe Asn Glu Val Gly Ala Asn Ala Val Thr Pro Met Thr
 60                  65                  70

CCA GCA CAA GCT GCA GAT AAA ATC CAT TCA TCC TTC CGC                 276
Pro Ala Gln Ala Ala Asp Lys Ile His Ser Ser Phe Arg
          75                  80                  85

TCT CTC AGC TCT GCA ATC AAT GCA TCC ACA GGG AAT TAT                 315
Ser Leu Ser Ser Ala Ile Asn Ala Ser Thr Gly Asn Tyr
                    90                  95

TTA CTG GAA AGT GTC AAT AAG CTG TTT GGT GAG AAG TCT                 354
Leu Leu Glu Ser Val Asn Lys Leu Phe Gly Glu Lys Ser
     100                 105                 110

GCG AGC TTC CGG GAA GAA TAT ATT CGA CTC TGT CAG AAA                 393
Ala Ser Phe Arg Glu Glu Tyr Ile Arg Leu Cys Gln Lys
               115                 120

TAT TAC TCC TCA GAA CCC CAG GCA GTA GAC TTC CTA GAA                 432
Tyr Tyr Ser Ser Glu Pro Gln Ala Val Asp Phe Leu Glu
125                 130                 135

TGT GCA GAA GAA GCT AGA AAA AAG ATT AAT TCC TGG GTC                 471
Cys Ala Glu Glu Ala Arg Lys Lys Ile Asn Ser Trp Val
          140                 145                 150
```

```
AAG ACT CAA ACC AAA GGC AAA ATC CCA AAC TTG TTA CCT  510
Lys Thr Gln Thr Lys Gly Lys Ile Pro Asn Leu Leu Pro
                    155                 160

GAA GGT TCT GTA GAT GGG GAT ACC AGG ATG GTC CTG GTG  549
Glu Gly Ser Val Asp Gly Asp Thr Arg Met Val Leu Val
    165                 170                 175

AAT GCT GTC TAC TTC AAA GGA AAG TGG AAA ACT CCA TTT  588
Asn Ala Val Tyr Phe Lys Gly Lys Trp Lys Thr Pro Phe
            180                 185

GAG AAG AAA CTA AAT GGG CTT TAT CCT TTC CGT GTA AAC  627
Glu Lys Lys Leu Asn Gly Leu Tyr Pro Phe Arg Val Asn
190                 195                 200

TCG GCT CAG CGC ACA CCT GTA CAG ATG ATG TAC TTG CGT  666
Ser Ala Gln Arg Thr Pro Val Gln Met Met Tyr Leu Arg
        205                 210                 215

GAA AAG CTA AAC ATT GGA TAC ATA GAA GAC CTA AAG GCT  705
Glu Lys Leu Asn Ile Gly Tyr Ile Glu Asp Leu Lys Ala
                220                 225

CAG ATT CTA GAA CTC CCA TAT GCT GGA GAT GTT AGC ATG  744
Gln Ile Leu Glu Leu Pro Tyr Ala Gly Asp Val Ser Met
    230                 235                 240

TTC TTG TTG CTT CCA GAT GAA ATT GCC GAT GTG TCC ACT  783
Phe Leu Leu Leu Pro Asp Glu Ile Ala Asp Val Ser Thr
            245                 250

GGC TTG GAG CTG CTG GAA AGT GAA ATA ACC TAT GAC AAA  822
Gly Leu Glu Leu Leu Glu Ser Glu Ile Thr Tyr Asp Lys
255                 260                 265

CTC AAC AAG TGG ACC AGC AAA GAC AAA ATG GCT GAA GAT  861
Leu Asn Lys Trp Thr Ser Lys Asp Lys Met Ala Glu Asp
        270                 275                 280

GAA GTT GAG GTA TAC ATA CCC CAG TTC AAA TTA GAA GAG  900
Glu Val Glu Val Tyr Ile Pro Gln Phe Lys Leu Glu Glu
                285                 290

CAT TAT GAA CTC AGA TCC ATT CTG AGA AGC ATG GGC ATG  939
His Tyr Glu Leu Arg Ser Ile Leu Arg Ser Met Gly Met
    295                 300                 305
```

```
GAG GAC GCC TTC AAC AAG GGA CGG GCC AAT TTC TCA GGG  978
Glu Asp Ala Phe Asn Lys Gly Arg Ala Asn Phe Ser Gly
            310                 315

ATG TCG GAG AGG AAT GAC CTG TTT CTT TCT GAA GTG TTC 1017
Met Ser Glu Arg Asn Asp Leu Phe Leu Ser Glu Val Phe
320                 325                 330

CAC CAA GCC ATG GTG GAT GTG AAT GAG GAG GGC ACT GAA 1056
His Gln Ala Met Val Asp Val Asn Glu Glu Gly Thr Glu
        335                 340                 345

GCA GCC GCT GGC ACA GGA GGT GTT ATG ACA GGG AGA ACT 1095
Ala Ala Ala Gly Thr Gly Gly Val Met Thr Gly Arg Thr
                350                 355

GGA CAT GGA GGC CCA CAG TTT GTG GCA GAT CAT CCT TTT 1134
Gly His Gly Gly Pro Gln Phe Val Ala Asp His Pro Phe
    360                 365                 370

CTT TTT CTT ATT ATG CAT AAG ATA ACC AAC TGC ATT TTA 1173
Leu Phe Leu Ile Met His Lys Ile Thr Asn Cys Ile Leu
            375                 380

TTT TTC GGC AGA TTT TCC TCA CCC TAA AACTAAGCGT      1210
Phe Phe Gly Arg Phe Ser Ser Pro
385                 390

GCTGCTTCTG CAAAAGATTT TTGTAGATGA GCTGTGTGCC         1250

TCAGAATTGC TATTTCAAAT TGCCAAAAAT TTAGAGATGT         1290

TTTCTACATA TTTCTGCTCT TCTGAACAAC TTCTGCTACC         1330

CACTAAATAA AAACACAGAA ATAATTAGAC AATTGTCTAT         1370

TATAACATGA CAACCCTATT AATCATTTGG TCTTCTAAAA         1410

TGGGATCATG CCCATTTAGA TTTTCCTTAC TATCAGTTTA         1450

TTTTTATAAC ATTAACTTTT ACTTTGTTAT TTATTATTTT         1490

ATATAATGGT GAGTTTTTGG GG                           1512
```

INFORMATION FOR SEQ ID NO. 3

(i)	SEQUENCE CHARACTERISTICS	
(A)	LENGTH:	1482 base pairs
(B)	TYPE:	Nucleic acid
(C)	STRANDEDNESS:	Double stranded
(D)	TOPOLOGY:	Linear
(ii)	MOLECULE TYPE:	cDNA TO mRNA
(A)	DESCRIPTION:	Codes for human plasminogen activator inhibitor type 2 protein in which amino acids 66 to 98 inclusive have been deleted.
(iii)	HYPOTHETICAL:	No
(iv)	ANTI-SENSE:	No
(v)	FRAGMENT TYPE:	N/A
(vi)	ORIGINAL SOURCE:	
(A)	ORGANISM:	Homo sapiens
(B)	STRAIN:	
(C)	INDIVIDUAL ISOLATE:	
(D)	DEVELOPMENT STAGE:	
(E)	HAPLOTYPE:	
(F)	TISSUE TYPE:	
(G)	CELL TYPE:	Monocyte
(H)	CELL LINE:	U937
(I)	ORGANELLE:	
(vii)	IMMEDIATE SOURCE:	
(A)	LIBRARY:	
(B)	CLONE:	BTA 1922

(ix) FEATURE:

(A) NAME/KEY: PAI-2 variant

(B) LOCATION: amino acids 66-98 inclusive deleted

(C) IDENTIFICATION METHOD: By experiment

(D) OTHER INFORMATION: Removes a protease sensitive site, product binds to urokinase, tissue plasminogen activator

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 3

```
GATCTGTAAG GAGGTATATA A ATG GAG GAT CTT TGT GTG GCA   42
                        Met Glu Asp Leu Cys Val Ala
                                                5

AAC ACA CTC TTT GCC CTC AAT TTA TTC AAG CAT CTG GCA   81
Asn Thr Leu Phe Ala Leu Asn Leu Phe Lys His Leu Ala
          10                  15                  20

AAA GCA AGC CCC ACC CAG AAC CTC TTC CTC TCC CCA TGG  120
Lys Ala Ser Pro Thr Gln Asn Leu Phe Leu Ser Pro Trp
                    25                  30

AGC ATC TCG TCC ACC ATG GCC ATG GTC TAC ATG GGC TCC  159
Ser Ile Ser Ser Thr Met Ala Met Val Tyr Met Gly Ser
     35                  40                  45

AGG GGC AGC ACC GAA GAC CAG ATG GCC AAG GTG CTT CAG  198
Arg Gly Ser Thr Glu Asp Gln Met Ala Lys Val Leu Gln
                50                  55

TTT AAT GAA GTG GGA GCC GCT GCA GAT AAA ATC CAT TCA  237
Phe Asn Glu Val Gly Ala Ala Ala Asp Lys Ile His Ser
 60                  65                  70

TCC TTC CGC TCT CTC AGC TCT GCA ATC AAT GCA TCC ACA  276
Ser Phe Arg Ser Leu Ser Ser A8a Ile Asn Ala Ser Thr
          75                  80                  85


GGG AAT TAT TTA CTG GAA AGT GTC AAT AAG CTG TTT GGT  315
Gly Asn Tyr Leu Leu Glu Ser Val Asn Lys Leu Phe Gly
                    90                  95

GAG AAG TCT GCG AGC TTC CGG GAA GAA TAT ATT CGA CTC  354
Glu Lys Ser Ala Ser Phe Arg Glu Glu Tyr Ile Arg Leu
     100                 105                 110

TGT CAG AAA TAT TAC TCC TCA GAA CCC CAG GCA GTA GAC  393
Cys Gln Lys Tyr Tyr Ser Ser Glu Pro Gln Ala Val Asp
                115                 120

TTC CTA GAA TGT GCA GAA GAA GCT AGA AAA AAG ATT AAT  432
Phe Leu Glu Cys Ala Glu Glu Ala Arg Lys Lys Ile Asn
125                 130                 135
```

```
TCC TGG GTC AAG ACT CAA ACC AAA GGC AAA ATC CCA AAC                471
Ser Trp Val Lys Thr Gln Thr Lys Gly Lys Ile Pro Asn
    140                 145                 150

TTG TTA CCT GAA GGT TCT GTA GAT GGG GAT ACC AGG ATG                510
Leu Leu Pro Glu Gly Ser Val Asp Gly Asp Thr Arg Met
                155                 160

GTC CTG GTG AAT GCT GTC TAC TTC AAA GGA AAG TGG AAA                549
Val Leu Val Asn Ala Val Tyr Phe Lys Gly Lys Trp Lys
    165                 170                 175

ACT CCA TTT GAG AAG AAA CTA AAT GGG CTT TAT CCT TTC                588
Thr Pro Phe Glu Lys Lys Leu Asn Gly Leu Tyr Pro Phe
            180                 185

CGT GTA AAC TCG GCT CAG CGC ACA CCT GTA CAG ATG ATG                627
Arg Val Asn Ser Ala Gln Arg Thr Pro Val Gln Met Met
190                 195                 200

TAC TTG CGT GAA AAG CTA AAC ATT GGA TAC ATA GAA GAC                666
Tyr Leu Arg Glu Lys Leu Asn Ile Gly Tyr Ile Glu Asp
        205                 210                 215

CTA AAG GCT CAG ATT CTA GAA CTC CCA TAT GCT GGA GAT                705
Leu Lys Ala Gln Ile Leu Glu Leu Pro Tyr Ala Gly Asp
                220                 225

GTT AGC ATG TTC TTG TTG CTT CCA GAT GAA ATT GCC GAT                744
Val Ser Met Phe Leu Leu Leu Pro Asp Glu Ile Ala Asp
    230                 235                 240

GTG TCC ACT GGC TTG GAG CTG CTG GAA AGT GAA ATA ACC                783
Val Ser Thr Gly Leu Glu Leu Leu Glu Ser Glu Ile Thr
            245                 250

TAT GAC AAA CTC AAC AAG TGG ACC AGC AAA GAC AAA ATG                822
Tyr Asp Lys Leu Asn Lys Trp Thr Ser Lys Asp Lys Met
255                 260                 265

GCT GAA GAT GAA GTT GAG GTA TAC ATA CCC CAG TTC AAA                861
Ala Glu Asp Glu Val Glu Val Tyr Ile Pro Gln Phe Lys
        270                 275                 280

TTA GAA GAG CAT TAT GAA CTC AGA TCC ATT CTG AGA AGC                900
Leu Glu Glu His Tyr Glu Leu Arg Ser Ile Leu Arg Ser
                285                 290
```

```
ATG GGC ATG GAG GAC GCC TTC AAC AAG GGA CGG GCC AAT  939
Met Gly Met Glu Asp Ala Phe Asn Lys Gly Arg Ala Asn
    295                 300                 305

TTC TCA GGG ATG TCG GAG AGG AAT GAC CTG TTT CTT TCT  978
Phe Ser Gly Met Ser Glu Arg Asn Asp Leu Phe Leu Ser
            310                 315

GAA GTG TTC CAC CAA GCC ATG GTG GAT GTG AAT GAG GAG 1017
Glu Val Phe His Gln Ala Met Val Asp Val Asn Glu Glu
320                 325                 330

GGC ACT GAA GCA GCC GCT GGC ACA GGA GGT GTT ATG ACA 1056
Gly Thr Glu Ala Ala Ala Gly Thr Gly Gly Val Met Thr
        335                 340                 345

GGG AGA ACT GGA CAT GGA GGC CCA CAG TTT GTG GCA GAT 1095
Gly Arg Thr Gly His Gly Gly Pro Gln Phe Val Ala Asp
                350                 355

CAT CCT TTT CTT TTT CTT ATT ATG CAT AAG ATA ACC AAC 1134
His Pro Phe Leu Phe Leu Ile Met His Lys Ile Thr Asn
    360                 365                 370

TGC ATT TTA TTT TTC GGC AGA TTT TCC TCA CCC TAA     1170
Cys Ile Leu Phe Phe Gly Arg Phe Ser Ser Pro  .
            375                 380

AACTAAGCGT GCTGCTTCTG CAAAAGATTT TTGTAGATGA         1210

GCTGTGTGCC TCAGAATTGC TATTTCAAAT TGCCAAAAAT         1250

TTAGAGATGT TTTCTACATA TTTCTGCTCT TCTGAACAAC         1290

TTCTGCTACC CACTAAATAA AAACACAGAA ATAATTAGAC         1330

AATTGTCTAT TATAACATGA CAACCCTATT AATCATTTGG         1370

TCTTCTAAAA TGGGATCATG CCCATTTAGA TTTTCCTTAC         1410

TATCAGTTTA TTTTTATAAC ATTAACTTTT ACTTTGTTAT         1450

TTATTATTTT ATATAATGGT GAGTTTTTGG GG                 1482
```

INFORMATION FOR SEQUENCE ID NO. 4

(i)	SEQUENCE CHARACTERISTICS	
(A)	LENGTH:	27 base pairs
(B)	TYPE:	Nucleic acid
(C)	STRANDEDNESS:	Single
(D)	TOPOLOGY:	Linear
(ii)	MOLECULE TYPE:	Other nucleic acid
	DESCRIPTION:	Synthetic DNA oligonucleotide
(iii)	HYPOTHETICAL:	No
(iv)	ANTISENSE:	No
(xi)	SEQUENCE DESCRIPTION:	SEQ ID No. 4

GGCCCATATG ATATCTCGAG ACTAGTC 27

INFORMATION FOR SEQ ID NO: 5

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(A) DESCRIPTION: coding region from base 241 to 348 in PAI-2 molecule showing amino acids deleted in 66-98 amino acid deletion variant.

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(v) FRAGMENT TYPE: Internal

(xi) DESCRIPTION OF SEQUENCE: SEQUENCE ID NO. 5

GCC GCT GCA 9

Ala Ala Ala

INFORMATION FOR SEQ ID NO: 6

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA
(A) DESCRIPTION: coding region from base 241 to 348 in PAI-2 molecule showing amino acids deleted in 74-96 amino acid deletion variant.

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(v) FRAGMENT TYPE: Internal

(xi) DESCRIPTION OF SEQUENCE: SEQUENCE ID NO: 6

GCC AAT GCA GTT ACC CCC ATG ACT CCA GCA CAA GCT GCA 39
Ala Asn Ala Val Thr Pro Met Thr Pro Ala Gln Ala Ala
5 10

INFORMATION FOR SEQ ID NO: 7

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: synthetic DNA adaptor for replacing HinfI/PstI region of PAI-2 gene in 74-96 amino acid coding region deletion variant

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(v) FRAGMENT TYPE: Internal

(xi) DESCRIPTION OF SEQUENCE: SEQUENCE ID NO. 7

ACT CCA GCA CAA GCT GCA 18

Thr Pro Ala Gln Ala Ala

5

INFORMATION FOR SEQ ID NO: 8

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: complementary sequence to SEQ ID No. 7 adaptor for replacing HinfI/PstI region of PAI-2 gene in 74-96 amino acid coding region deletion variant

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: Yes

(v) FRAGMENT TYPE:

(xi) DESCRIPTION OF SEQUENCE: SEQUENCE ID NO. 8

GCT TGT GCT GG 11

INFORMATION FOR SEQ ID NO: 9

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: synthetic DNA oligonucleotide for use in PCR reaction to create gene encoding 66-98 amino acid deletion variant of PAI-2

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: Yes

(v) FRAGMENT TYPE:

(xi) DESCRIPTION OF SEQUENCE: SEQUENCE ID NO. 9

CCT CTT CTG CAG ATT CTA GGA A 22

INFORMATION FOR SEQ ID NO: 10

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: synthetic DNA oligonucleotide for use in PCR reaction to create gene encoding 66-98 amino acid deletion variant of PAI-2

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: Yes

(v) FRAGMENT TYPE:

(xi) DESCRIPTION OF SEQUENCE: SEQUENCE ID NO. 10

AT CTG CAG CGG CTC CCA CTT CAT TAA ACT 29

INFORMATION FOR SEQ ID NO: 11

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: synthetic DNA oligonucleotide for use in PCR reaction to create gene encoding 66-98 amino acid deletion variant of PAI-2

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(v) FRAGMENT TYPE:

(xi) DESCRIPTION OF SEQUENCE: SEQUENCE ID NO. 11

GTG GGA GCC GCT GCA GAT AAA ATC CAT T 28

INFORMATION FOR SEQUENCE ID NO. 12

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 27 base pairs
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION: Synthetic DNA oligonucleotide

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID No. 12

GATCTNNNNN NNNNNNNNNN NATGGAG 27

INFORMATION FOR SEQUENCE ID NO. 13

(i)	SEQUENCE CHARACTERISTICS	
(A)	LENGTH:	26 base pairs
(B)	TYPE:	Nucleic acid
(C)	STRANDEDNESS:	Single
(D)	TOPOLOGY:	Linear
(ii)	MOLECULE TYPE:	Other nucleic acid
(A)	DESCRIPTION:	Synthetic DNA oligonucleotide
(iii)	HYPOTHETICAL:	No
(iv)	ANTISENSE:	No

(xi) SEQUENCE DESCRIPTION: SEQ ID No. 13

GATCTNNNNN NNNNNNNNNN ATGGAG 26

INFORMATION FOR SEQUENCE ID NO. 14

(i)	SEQUENCE CHARACTERISTICS	
(A)	LENGTH:	15 amino acids
(B)	TYPE:	Amino acid
(C)	STRANDEDNESS:	
(D)	TOPOLOGY:	Linear
(ii)	MOLECULE TYPE:	Peptide
(iii)	HYPOTHETICAL:	No
(iv)	ANTISENSE:	No
(v)	FRAGMENT TYPE:	N-terminal fragment

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 14

Lys Gly Ser Tyr Pro Asp Ala Ile Leu Gln Ala Gln Ala Ala Asp
5 10 15

INFORMATION FOR SEQUENCE ID NO. 15

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 10 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(v) FRAGMENT TYPE: N-terminal fragment

(xi) SEQUENCE DESCRIPTION: SEQ ID No. 15

Phe Met Gln Gln Ile Gln Lys Gly Ser Tyr
5 10

INFORMATION FOR SEQUENCE ID NO. 16

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 15 amino acids

(B) TYPE: Amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(v) FRAGMENT TYPE: N terminal fragment

(xi) SEQUENCE DESCRIPTION: SEQ ID No. 16

Gly Phe Met Gln Gln Ile Gln Lys Gly Ser Tyr Pro Asp Ala Ile
5 10 15

INFORMATION FOR SEQ ID NO. 17

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 27 base pairs

(B) TYPE: Nucleotide

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION: Synthetic DNA oligonucleotide

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID No. 17

CAG	CAG	ATC	CAG	GCA	GGT	AGT	TAT	CCT	27
Gln	Gln	Ile	Gln	Ala	Gly	Ser	Tyr	Pro	

5

INFORMATION FOR SEQ ID NO: 18

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 108 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(A) DESCRIPTION: bases 241 to 348 of native PAI-2 coding sequence illustrating difference in this region for deletion variants of PAI-2

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(v) FRAGMENT TYPE:

(xi) DESCRIPTION OF SEQUENCE: SEQUENCE ID NO. 18

```
GCC AAT GCA GTT ACC CCC ATG ACT CCA GAG AAC TTT ACC AGC TGT      45
Ala Asn Ala Val Thr Pro Met Thr Pro Glu Asn Phe Thr Ser Cys
                5                   10                  15

GGG TTC ATG CAG CAG ATC CAG AAG GGT AGT TAT CCT GAT GCG ATT      90
Gly Phe Met Gln Gln Ile Gln Lys Gly Ser Tyr Pro Asp Ala Ile
                20                  25                  30

TTG CAG GCA CAA GCT GCA                                         108
Leu Gln Ala Gln Ala Ala
                35
```

CLAIMS

1. A PAI-2 variant in which the 66-98 amino acid residue region has been altered to eliminate at least one protease sensitive site, which variant maintains biological activity of PAI-2 and amino acids up to 65 and from 99 of PAI-2 in frame.

2. A PAI-2 variant according to claim 1 which variant is a deletion variant in which at least one amino residue in the 66-98 amino acid residue region has been deleted.

3. The PAI-2 variant Δ74-96 wherein Δ74-96 has amino acids 74-96 inclusive of PAI-2 deleted.

4. The PAI-2 variant Δ66-98, wherein Δ66-98 has amino acids 66-98 inclusive of PAI-2 deleted.

5. A PAI-2 variant according to any one of claims 1 to 4 in labelled form.

6. A PAI-2 variant in labelled form according to claim 5 wherein the label is selected from the group consisting of radioisotopes, enzymes and chemical agents.

7. A DNA molecule, the sequence of which encodes a PAI-2 variant according to any one of claims 1 to 4.

8. A recombinant DNA molecule comprising a DNA molecule according to claim 7 and vector DNA.

9. A recombinant DNA molecule according to claim 8 wherein the vector DNA is plasmid DNA.

10. A recombinant DNA molecule according to claim 9. wherein the plasmid DNA is selected from the group consisting of E. coli expression vectors, baculovirus transfer vectors, mammalian expression vectors, vaccinia virus expression vectors and retroviral expression vectors.

11. A recombinant DNA molecule according to claim 10 wherein the E. coli expression vector is selected from the group consisting of:

E. coli expression vectors based on the P_L promoter;
E. coli expression vectors based on the lac promoter;
E. coli expression vectors based on the tac promoter;
E. coli expression vectors based on the trp promoter;
pGEM4Z and plasmids derived therefrom; and
pSp70 and plasmids derived therefrom.

12. A recombinant DNA molecule according to claim 10 wherein the baculovirus transfer vector is selected from the group consisting of pAc373, pAc360 and plasmids derived therefrom.

13. A recombinant DNA molecule according to claim 10 wherein the mammalian expression vector is selected from the group consisting of:

pBPV-1; pBPV-BV1; pdBPV-MMTneo; SV40 based expression vectors including pBTA 613; and plasmids derived therefrom.

14. Recombinant DNA molecule pBTA 829 as hereinbefore defined.

15. Recombinant DNA molecule pBTA 840 as hereinbefore defined.

16. Recombinant DNA molecule pMINDEL 74-96 as hereinbefore defined.

17. A transformed host cell transformed by a recombinant DNA molecule according to claim 8.

18. A transformed host cell according to claim 17 wherein the host cell is selected from the group consisting of E. coli K12 strains, cells derived from eukaryotic organisms and cell lines derived from the insects Spodoptera frugiperda and Bombyx mori.

19. A transformed host cell according to claim 18 wherein the cells derived from eukaryotic organisms are selected from the group consisting of COS cells, CHO cells, U937 cells, BHK-21 cells, Vero cells, CV1 cells and C127 cells.

20. A process for producing a PAI-2 variant according to claim 1 which process comprises: deleting nucleotides from the 66-98 amino acid residue region of a DNA molecule encoding PAI-2 such that the amino acids up to residue 65 and from residue 99 of PAI-2 remain in frame and the variant maintains biological activity of PAI-2.

21. A process for producing a recombinant DNA molecule according to claim 8 which process comprises inserting a DNA molecule according to claim 7 into vector DNA.

22. A process for producing a transformed host according to claim 17 which process comprises making a suitable host cell competent for transformation, and transforming the competent host cell with a recombinant DNA molecule according to claim 8.

23. A therapeutic and/or diagnostic composition comprising an effective amount of at least one PAI-2 variant according to any one of claims 1 to 4 together with a pharmaceutically acceptable carrier, excipient, and/or diluent.

24. A therapeutic and/or diagnostic composition comprising an effective amount of at least one labelled variant according to claim 5 together with a pharmaceutically acceptable carrier, excipient, and/or diluent.

25. A method of inhibiting tumour invasion comprising administering to a patient requiring such treatment an effective amount of a PAI-2 variant according to claim 1 and/or a composition according to claim 23.

26. A method of treating a tumour which method comprises administering to a patient requiring such treatment an effective amount of a PAI-2 variant according to claim 1 and/or a composition according to claim 23.

27. A method of treatment of an inflammatory disease which method comprises administering to a patient requiring such treatment an effective amount of a PAI-2 variant according to claim 1 and/or a composition according to claim 23.

28. A method according to claim 27 wherein the inflammatory disease is selected from the group consisting of rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, ulcerative colitis, psoriasis and pemphigus.

29. A method of treating a fibrinolytic disorder comprising administering to a patient requiring such treatment, an effective amount of a PAI-2 variant according to claim 1 and/or a composition according to claim 23.

30. A method according to claim 29 wherein the fibrinolytic disorder is systemic fibrinolysis.

31. A method of treatment of a condition selected from the group consisting of multiple sclerosis, corneal ulceration, gastroduodenal ulceration, purpura, periodontitis, haemorrhage and muscular dystrophy which method comprises administering an effective amount of a PAI-2 variant according to claim 1 and/or a composition according to claim 23 to a patient in need of such treatment.

32. A method of locating and/or defining the boundaries of a tumour in a histological specimen or in vivo which method comprises applying a labelled PAI-2 variant according to claim 5 or a composition according to claim 24 to the specimen or administering the labelled PAI-2 variant or composition to a host in need of in vivo imaging and determining by imaging location of concentration of the label.

33. A method of improving clinical efficacy of plasminogen activator treatment of thrombosis which method comprises administering an effective amount of a PAI-2 variant according to claim 1/or a composition according to claim 23 to a host in need of such treatment, to counteract systemic activation of fibrinolysis and concomitant fibrin/fibrinogen breakdown.

34. An antibody against a PAI-2 variant according to any one of claims 1 to 4.

35. A polyclonal antibody according to claim 34.

36. A monoclonal antibody according to claim 34.

37. A process for preparing an antibody according to claim 34 which process comprises immunizing an immuno-competent host with an effective amount of a PAI-2 variant according to claim 1 and/or a composition according to claim 23.

38. An antibody composition comprising an antibody according to claim 34 together with a pharmaceutically acceptable carrier, diluent, and/or excipient.

39. A diagnostic reagent comprising an antibody according to claim 34 and/or an antibody composition according to claim 38.

40. A conjugate comprising a PAI-2 variant according to claim 1 linked to a cytotoxin.

41. A cytotoxin composition comprising a conjugate according to claim 40 together with a pharmaceutically acceptable carrier, diluent, and/or excipient.

42. A method fo delivering a cytotoxic agent to a tumour which method comprises administering an effective amount of a conjugate according to claim 40 and/or a cytotoxic composition according to claim 41 to a host in need of such treatment.

43. A diagnostic kit comprising a variant according to claim 1 and/or a composition according to claim 23 as standard, together with an antibody according to claim 34, an antibody composition according to claim 38 or a diagnostic reagent according to claim 39.

FIGURE 1 (SEQ ID NO. 1)

GTCAGACAGCAACTCAGAGAATAACCAGAGAACAACCAGATTGAAACA

49								75								102
ATG	GAG	GAT	CTT	TGT	GTG	GCA	AAC	ACA	CTC	TTT	GCC	CTC	AAT	TTA	TTC	AAG	CAT
MET	Glu	Asp	Leu	Cys	Val	Ala	Asn	Thr	Leu	Phe	Ala	Leu	Asn	Leu	Phe	Lys	His
1																	18
								129								156
CTG	GCA	AAA	GCA	AGC	CCC	ACC	CAG	AAC	CTC	TTC	CTC	TCC	CCA	TGG	AGC	ATC	TCG
Leu	Ala	Lys	Ala	Ser	Pro	Thr	Gln	Asn	Leu	Phe	Leu	Ser	Pro	Trp	Ser	Ile	Ser
19																	36
								183								210
TCC	ACC	ATG	GCC	ATG	GTC	TAC	ATG	GGC	TCC	AGG	GGC	AGC	ACC	GAA	GAC	CAG	ATG
Ser	Thr	MET	Ala	MET	Val	Tyr	MET	Gly	Ser	Arg	Gly	Ser	Thr	Glu	Asp	Gln	MET
37																	54
								237								264
GCC	AAG	GTG	CTT	CAG	TTT	AAT	GAA	GTG	GGA	GCC	AAT	GCA	GTT	ACC	CCC	ATG	ACT
Ala	Lys	Val	Leu	Gln	Phe	Asn	Glu	Val	Gly	Ala	Asn	Ala	Val	Thr	Pro	MET	Thr
55																	72
								291								318
CCA	GAG	AAC	TTT	ACC	AGC	TGT	GGG	TTC	ATG	CAG	CAG	ATC	CAG	AAG	GGT	AGT	TAT
Pro	Glu	Asn	Phe	Thr	Ser	Cys	Gly	Phe	MET	Gln	Gln	Ile	Gln	Lys	Gly	Ser	Tyr
73																	90
								345								372
CCT	GAT	GCG	ATT	TTG	CAG	GCA	CAA	GCT	GCA	GAT	AAA	ATC	CAT	TCA	TCC	TTC	CGC
Pro	Asp	Ala	Ile	Leu	Gln	Ala	Gln	Ala	Ala	Asp	Lys	Ile	His	Ser	Ser	Phe	Arg
91																	108

FIGURE 1 (Continued 1)

								399								426
TCT	CTC	AGC	TCT	GCA	ATC	AAT	GCA	TCC	ACA	GGG	AAT	TAT	TTA	CTG	GAA	AGT	GTC
Ser	Leu	Ser	Ser	Ala	Ile	Asn	Ala	Ser	Thr	Gly	Asn	Tyr	Leu	Leu	Glu	Ser	Val
109																126
								453								480
AAT	AAG	CTG	TTT	GGT	GAG	AAG	TCT	GCG	AGC	TTC	CGG	GAA	GAA	TAT	ATT	CGA	CTC
Asn	Lys	Leu	Phe	Gly	Glu	Lys	Ser	Ala	Ser	Phe	Arg	Glu	Glu	Tyr	Ile	Arg	Leu
127																144
								507								534
TGT	CAG	AAA	TAT	TAC	TCC	TCA	GAA	CCC	CAG	GCA	GTA	GAC	TTC	CTA	GAA	TGT	GCA
Cys	Gln	Lys	Tyr	Tyr	Ser	Ser	Glu	Pro	Gln	Ala	Val	Asp	Phe	Leu	Glu	Cys	Ala
145																162
								561								588
GAA	GAA	GCT	AGA	AAA	AAG	ATT	AAT	TCC	TGG	GTC	AAG	ACT	CAA	ACC	AAA	GGC	AAA
Glu	Glu	Ala	Arg	Lys	Lys	Ile	Asn	Ser	Trp	Val	Lys	Thr	Gln	Thr	Lys	Gly	Lys
163																180
								615								642
ATC	CCA	AAC	TTG	TTA	CCT	GAA	GGT	TCT	GTA	GAT	GGG	GAT	ACC	AGG	ATG	GTC	CTG
Ile	Pro	Asn	Leu	Leu	Pro	Glu	Gly	Ser	Val	Asp	Gly	Asp	Thr	Arg	MET	Val	Leu
181																198
								669								696
GTG	AAT	GCT	GTC	TAC	TTC	AAA	GGA	AAG	TGG	AAA	ACT	CCA	TTT	GAG	AAG	AAA	CTA
Val	Asn	Ala	Val	Tyr	Phe	Lys	Gly	Lys	Trp	Lys	Thr	Pro	Phe	Glu	Lys	Lys	Leu
199																216
								723								750
AAT	GGG	CTT	TAT	CCT	TTC	CGT	GTA	AAC	TCG	GCT	CAG	CGC	ACA	CCT	GTA	CAG	ATG
Asn	Gly	Leu	Tyr	Pro	Phe	Arg	Val	Asn	Ser	Ala	Gln	Arg	Thr	Pro	Val	Gln	MET
217																234

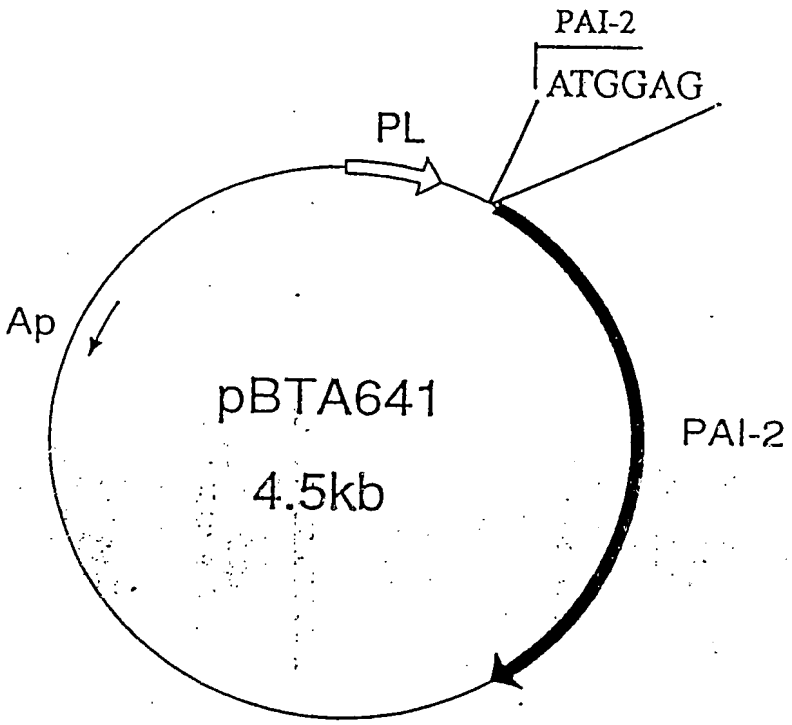
FIGURE 1 (Continued 2)

								777								804
ATG	TAC	TTG	CGT	GAA	AAG	CTA	AAC	ATT	GGA	TAC	ATA	GAA	GAC	CTA	AAG	GCT	CAG
MET	Tyr	Leu	Arg	Glu	Lys	Leu	Asn	Ile	Gly	Tyr	Ile	Glu	Asp	Leu	Lys	Ala	Gln
235																	252
								831								858
ATT	CTA	GAA	CTC	CCA	TAT	GCT	GGA	GAT	GTT	AGC	ATG	TTC	TTG	TTG	CTT	CCA	GAT
Ile	Leu	Glu	Leu	Pro	Tyr	Ala	Gly	Asp	Val	Ser	MET	Phe	Leu	Leu	Leu	Pro	Asp
253																	270
								885								912
GAA	ATT	GCC	GAT	GTG	TCC	ACT	GGC	TTG	GAG	CTG	CTG	GAA	AGT	GAA	ATA	ACC	TAT
Glu	Ile	Ala	Asp	Val	Ser	Thr	Gly	Leu	Glu	Leu	Leu	Glu	Ser	Glu	Ile	Thr	Tyr
271																	288
								939								966
GAC	AAA	CTC	AAC	AAG	TGG	ACC	AGC	AAA	GAC	AAA	ATG	GCT	GAA	GAT	GAA	GTT	GAG
Asp	Lys	Leu	Asn	Lys	Trp	Thr	Ser	Lys	Asp	Lys	MET	Ala	Glu	Asp	Glu	Val	Glu
289																	306
								993								1020
GTA	TAC	ATA	CCC	CAG	TTC	AAA	TTA	GAA	GAG	CAT	TAT	GAA	CTC	AGA	TCC	ATT	CTG
Val	Tyr	Ile	Pro	Gln	Phe	Lys	Leu	Glu	Glu	His	Tyr	Glu	Leu	Arg	Ser	Ile	Leu
307																	324
								1047								1074
AGA	AGC	ATG	GGC	ATG	GAG	GAC	GCC	TTC	AAC	AAG	GGA	CGG	GCC	AAT	TTC	TCA	GGG
Arg	Ser	MET	Gly	MET	Glu	Asp	Ala	Phe	Asn	Lys	Gly	Arg	Ala	Asn	Phe	Ser	Gly
325																	342
								1101								1128
ATG	TCG	GAG	AGG	AAT	GAC	CTG	TTT	CTT	TCT	GAA	GTG	TTC	CAC	CAA	GCC	ATG	GTG
MET	Ser	Glu	Arg	Asn	Asp	Leu	Phe	Leu	Ser	Glu	Val	Phe	His	Gln	Ala	MET	Val
343																	360

FIGURE 1 (Continued 3)

```
                                        1155                                                      1182
GAT  GTG  AAT  GAG  GAG  GGC  ACT  GAA  GCA  GCC  GCT  GGC  ACA  GGA  GGT  GTT  ATG  ACA
Asp  Val  Asn  Glu  Glu  Gly  Thr  Glu  Ala  Ala  Ala  Gly  Thr  Gly  Gly  Val  MET  Thr
361                                                                                   378

                                        1209                                                      1236
GGG  AGA  ACT  GGA  CAT  GGA  GGC  CCA  CAG  TTT  GTG  GCA  GAT  CAT  CCT  TTT  CTT  TTT
Gly  Arg  Thr  Gly  His  Gly  Gly  Pro  Gln  Phe  Val  Ala  Asp  His  Pro  Phe  Leu  Phe
379                                                                                   396

                                        1263                                                      1290
CTT  ATT  ATG  CAT  AAG  ATA  ACC  AAC  TGC  ATT  TTA  TTT  TTC  GGC  AGA  TTT  TCC  TCA
Leu  Ile  MET  His  Lys  Ile  Thr  Asn  Cys  Ile  Leu  Phe  Phe  Gly  Arg  Phe  Ser  Ser
397                                                                                   414

                                                                   1359
CCC  TAA  AACTAAGCGTGCTGCTTCTGCAAAAGATTTTTGTAGATGAGCTGTGTGCCTCAGAATTGCTAT
PRO   *
415

                                                                   1430
TTCAAATTGCCAAAAATTTAGAGATGTTTTCTACATATTTCTGCTCTTCTGAACAACTTCTGCTACCCACT

                                                                   1501
AAATAAAAACACAGAAATAATTAGACAATTGTCTATTATAACATGACAACCCTATTAATCATTTGGTCTTC

                                                                   1572
TAAAATGGGATCATGCCCATTTAGATTTTCCTTACTATCAGTTTATTTTTATAACATTAACTTTTACTTTG

                                 1610
TTATTTATTATTTTATATAATGGTGAGTTTTTAAATTA
```

FIGURE 2


PAI-2
ATGGAG
PL
Ap
pBTA641
4.5kb
PAI-2

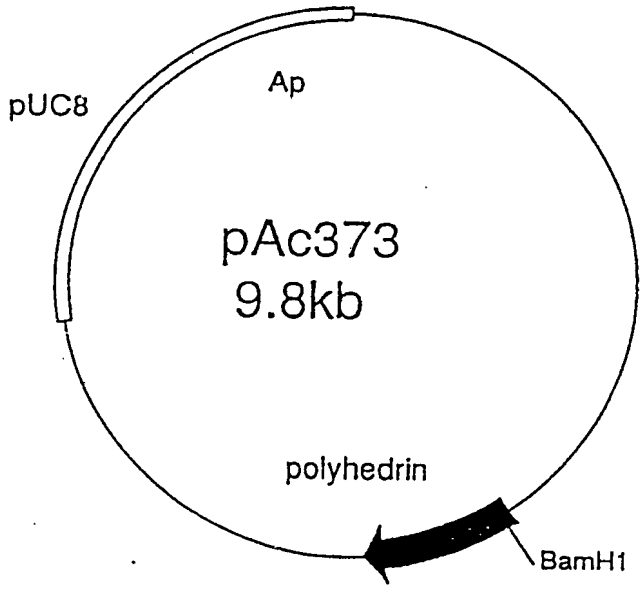
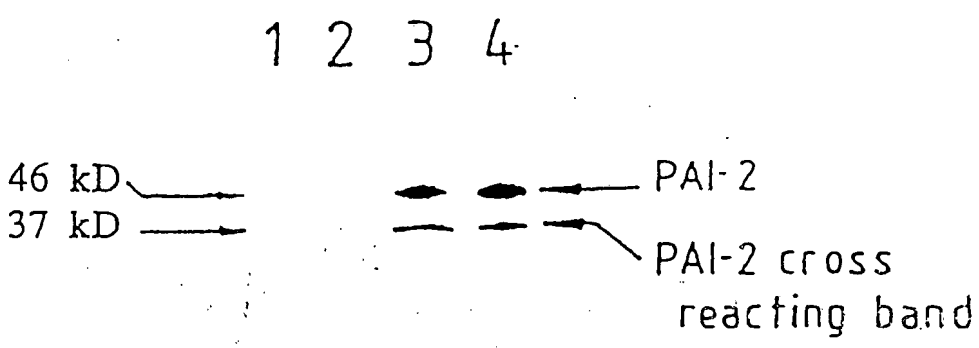

pUC8
Ap
pAc373
9.8kb
polyhedrin
BamH1

Fig.3



SUBSTITUTE SHEET

FIGURE 4

NATIVE PAI-2

```
241                                                                                                  292
|                                                                                                    |
GCC AAT GCA GTT ACC CCC ATG ACT CCA GAG AAC TTT ACC AGC TGT GGG TTC ATG (SEQ ID No. 18)
Ala Asn Ala Val Thr Pro Met Thr Pro Glu Asn Phe Thr Ser Cys Gly Phe Met
65                                                                                                   82

295                                                                                                  346
|                                                                                                    |
CAG CAG ATC CAG AAG GGT AGT TAT CCT GAT GCG ATT TTG CAG GCA CAA GCT GCA
Gln Gln Ile Gln Lys Gly Ser Tyr Pro Asp Ala Ile Leu Gln Ala Gln Ala Ala
83                                                                                                   100
```

Δ 74-96 PAI-2

```
241                                                  265  337              346
|                                                    |    |                |
GCC AAT GCA GTT ACC CCC ATG ACT CCA GCA CAA GCT GCA (SEQ ID No. 6)
Ala Asn Ala Val Thr Pro Met Thr Pro Ala Gln Ala Ala
65                                                   73   97               100
```

Δ 66-98 PAI-2

```
241  343  346
|    |    |
GCC GCT GCA (SEQ ID No. 5)
Ala Ala Ala
65  99  100
```

FIGURE 5

Oligo A_1 (18 mer) - Adaptor for Replacing HinfI/PstI region of PAI-2 Gene

5' - ACT CCA GCA CAA GCT GCA - 3'

Oligo A_2 (11 mer) - Complementary to A_1

5' - GCT TGT GCT GG - 3'

Oligos A_1/A_2 - After Kinasing, mixing and annealing

HinfI Co-hesive End						PstI Co-hesive End	
5' - ACT	CCA	GCA	CAA	GCT	GCA	- 3'	(SEQ ID No. 7)
3' -	GGT	CGT	GTT	CG		- 5'	(SEQ ID No. 8)
Thr	Pro	Ala	Gln	Ala	Ala	- encoded amino acids	
72	73	97	98	99	100	- position in native PAI-2 protein	

FIGURE 6

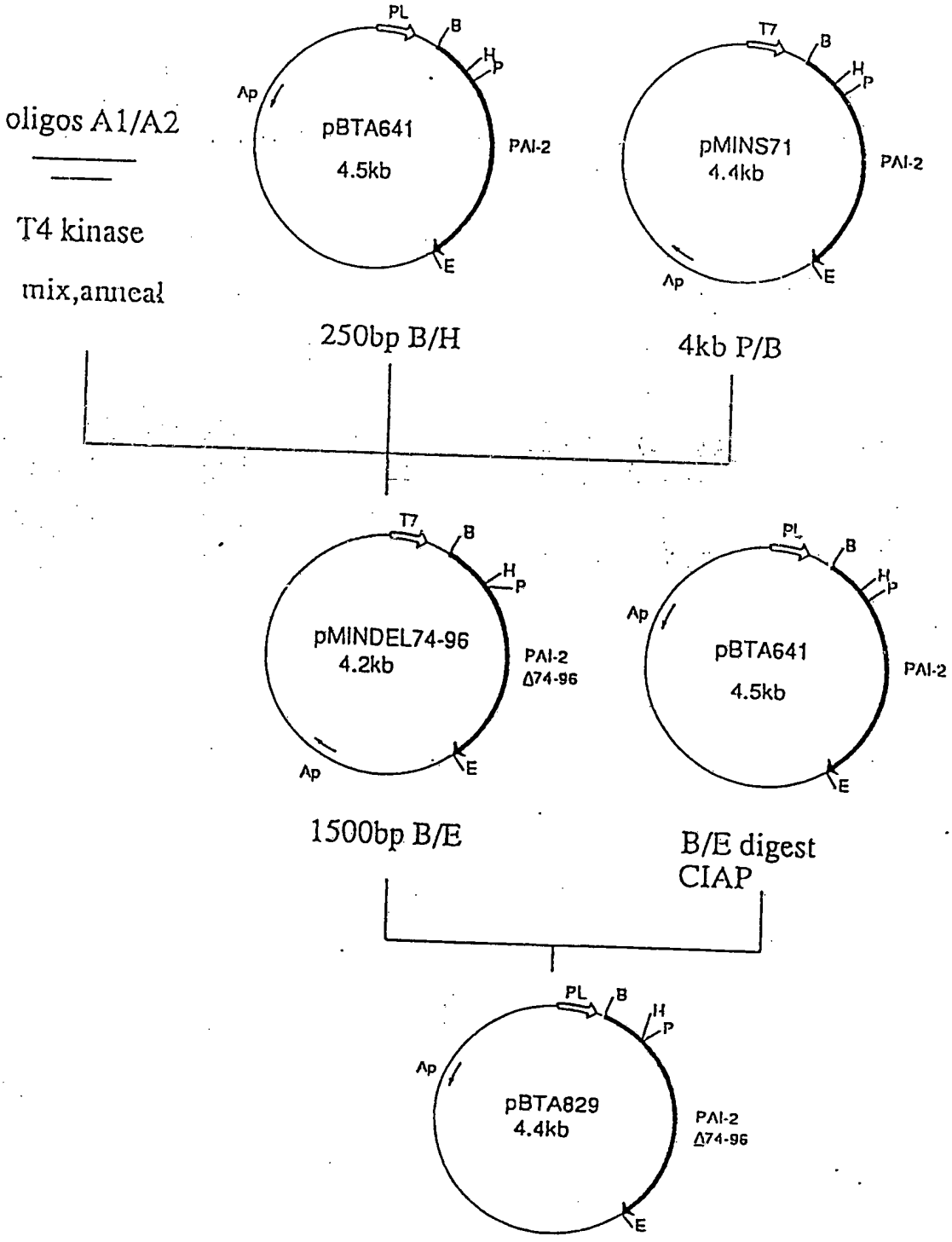
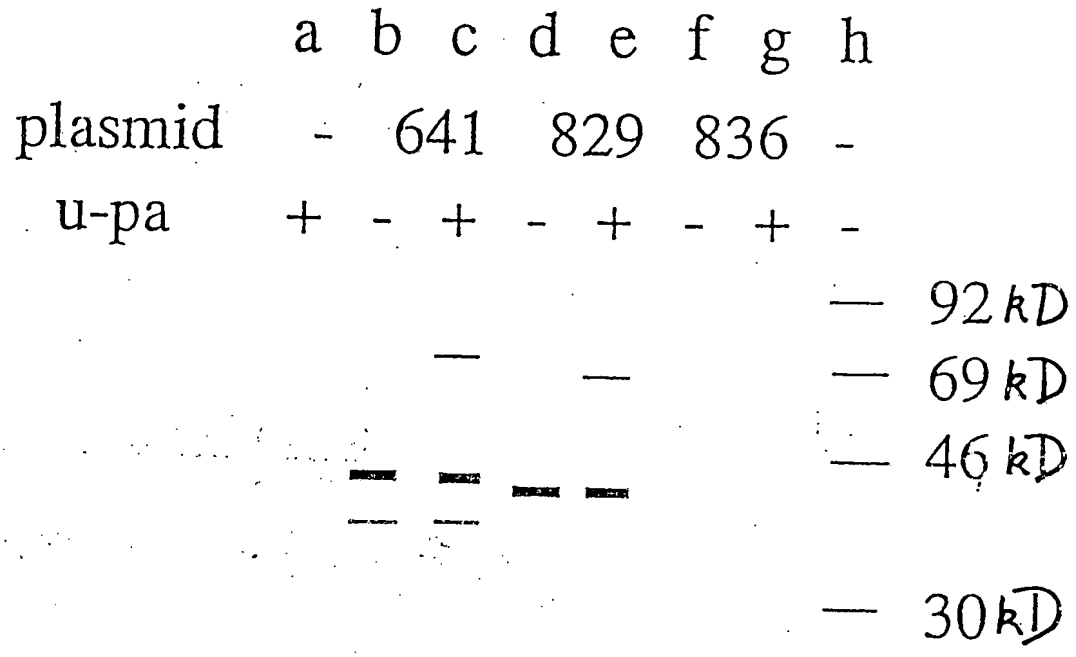

oligos A1/A2
T4 kinase
mix,anneal
pBTA641
4.5kb
PAI-2
pMINS71
4.4kb
PAI-2
250bp B/H
4kb P/B
pMINDEL74-96
4.2kb
PAI-2
Δ74-96
pBTA641
4.5kb
PAI-2
1500bp B/E
B/E digest
CIAP
pBTA829
4.4kb
PAI-2
Δ74-96

FIGURE 7

A.


a b c d e f g h
plasmid - 641 829 836 -
u-pa + - + - + - + -
92 kD
69 kD
46 kD
30 kD

B.

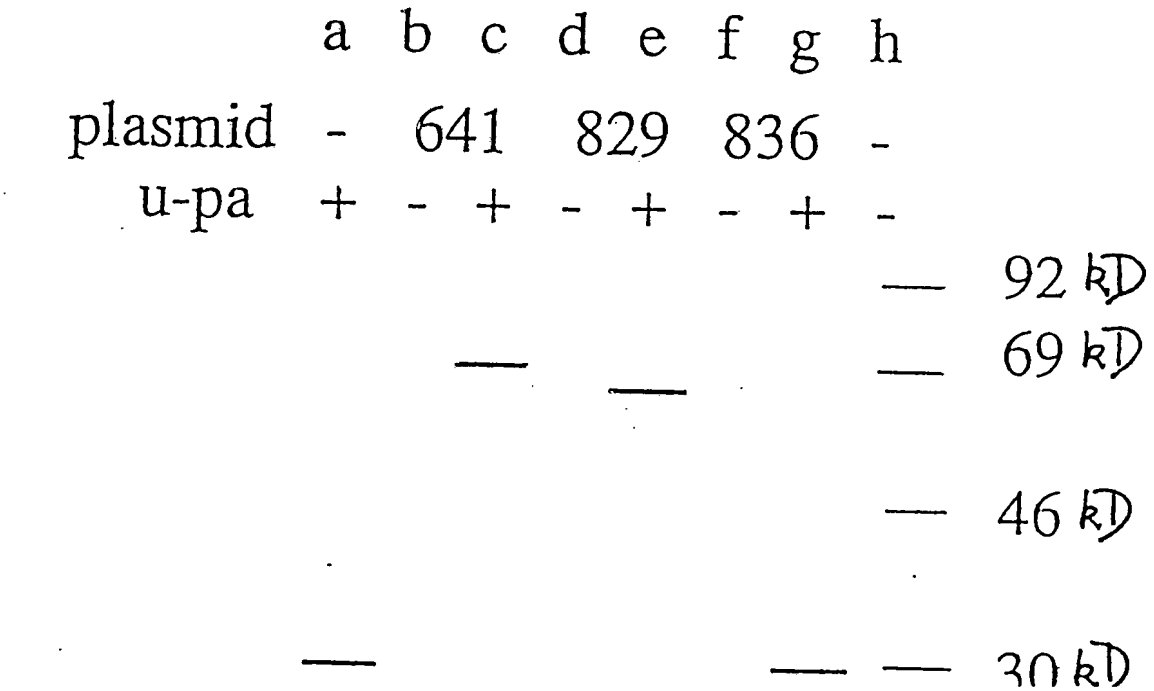

a b c d e f g h
plasmid - 641 829 836 -
u-pa + - + - + - + -
92 kD
69 kD
46 kD
30 kD

FIGURE 8

A134/301 (22mer) - oligo for PCR reaction, noncoding strand

```
      541                                 520
      |                                   |
5'- CTT CTT CTG CAG ATT CTA GGA A -3'   (SEQ ID NO. 9)
```

A134/304 (29mer) - oligo for PCR reaction, noncoding strand

```
      351          343 243                      224
      |            | |                          |
5'- ATC TG CAG CGG CTC CCA GTT CAT TAA ACT -3'  (SEQ ID NO. 10)
```

A134/305 (28mer) - oligo for PCR reaction, coding strand

```
    235      243 343                          361
    |          | |                            |
5'-GTG GGA GCC GCT GCA GAT AAA ATC CAT T -3'  (SEQ ID NO. 11)
```

FIGURE 9

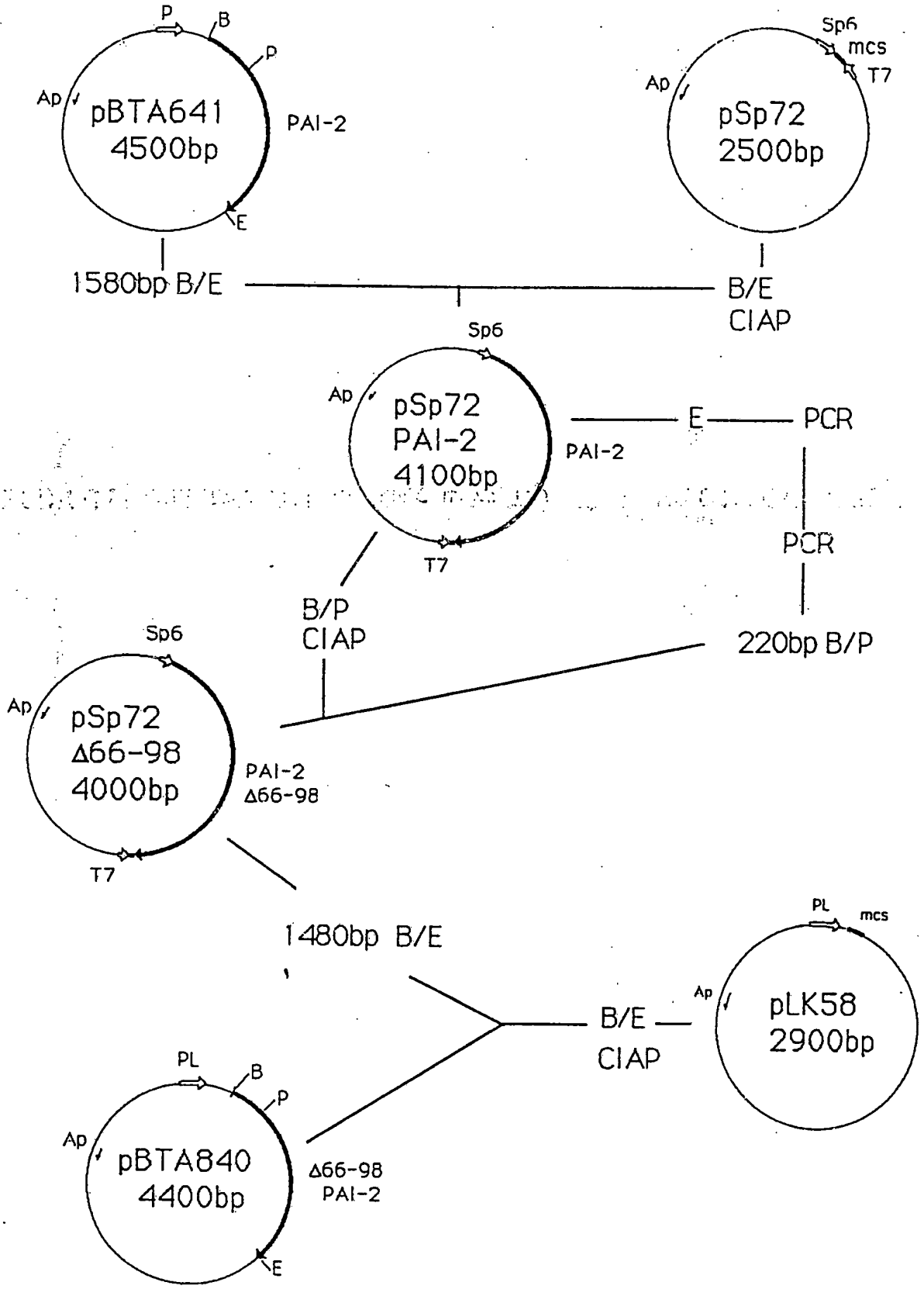
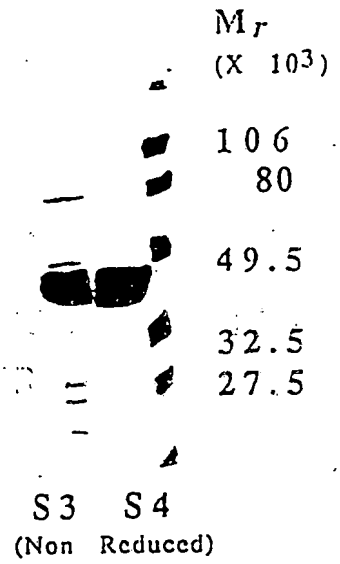

P
B
P
Ap
pBTA641
4500bp
PAI-2
E
1580bp B/E
Sp6
mcs
T7
Ap
pSp72
2500bp
B/E
CIAP
Sp6
Ap
pSp72
PAI-2
4100bp
PAI-2
T7
E
PCR
PCR
B/P
CIAP
220bp B/P
Sp6
Ap
pSp72
Δ66-98
4000bp
PAI-2
Δ66-98
T7
1480bp B/E
PL
mcs
Ap
pLK58
2900bp
B/E
CIAP
PL
B
P
Ap
pBTA840
4400bp
Δ66-98
PAI-2
E

Figure 10

a.


Mr
(X 10³)
106
80
49.5
32.5
27.5
S3 S4
(Non Reduced)

b.

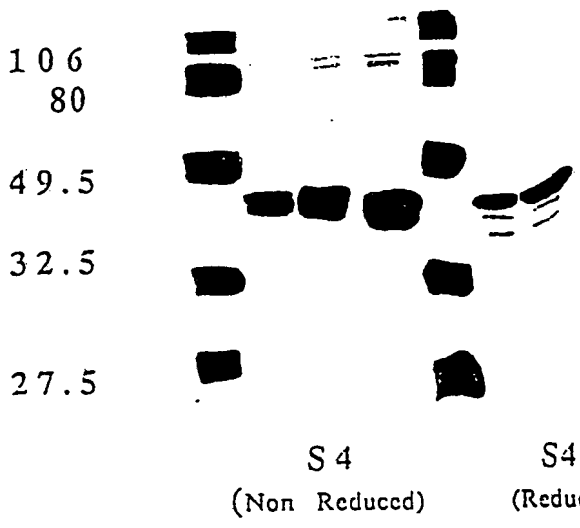
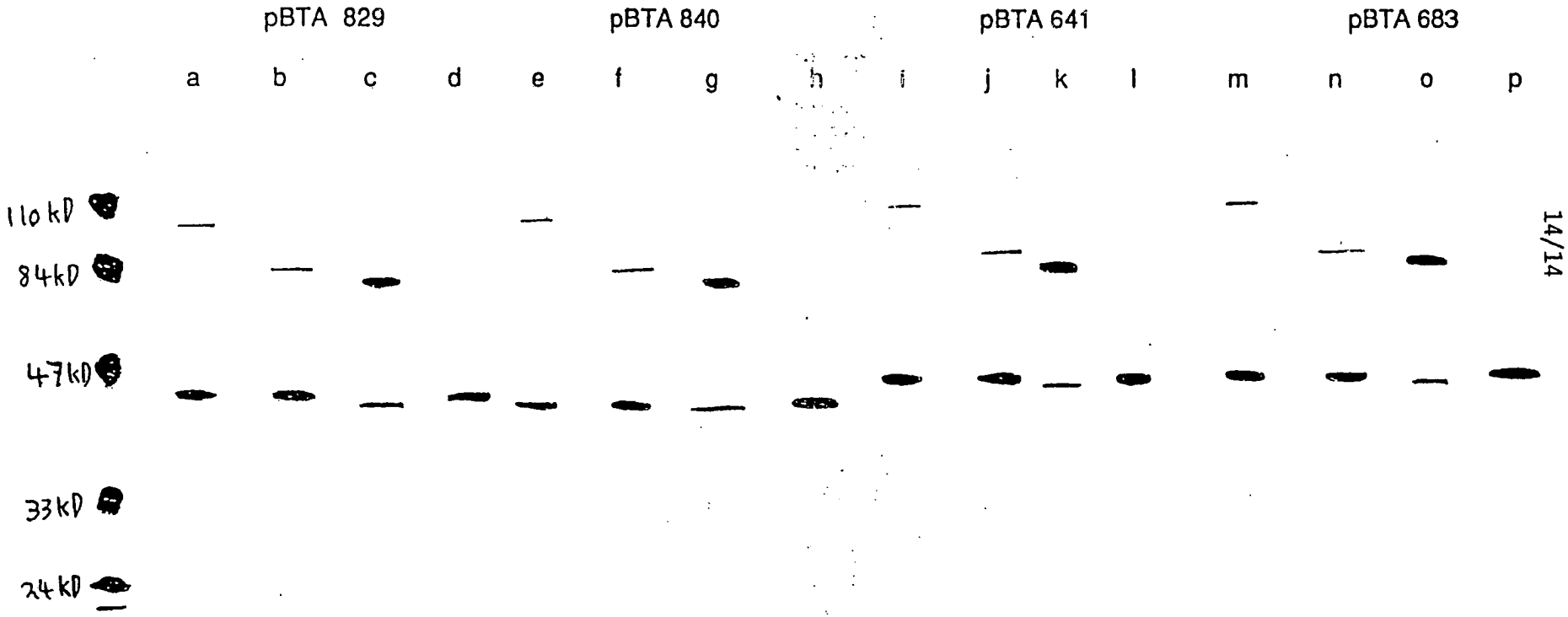

Mr
(X 10³)
106
80
49.5
32.5
27.5
S4
(Non Reduced)
S4
(Reduced)

FIGURE 11



Lanes a, e, i, m : PAI-2s plus single chain t-PA

Lanes b, f, j, n : PAI-2s plus two chain t-PA

Lanes c, g, k, o : PAI-2s plus U-PA

Lanes d, h, l, p : PAI-2s

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[5] C12N 15/15, C07K 13/00, G01N 33/574, G01N 33/53, C12P 21/08, C07K 15/12, A61K 37/64, A61K 49/02, A61K 47/48

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC	C12N 15/15, C07K 13/00, G01N 33/574, G01N 33/53, C12P 21/08, C07K 15/12

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

AU: IPC as above, Jopal, Chem Abs, Biosis (Keywords) Plasminogen Activator Inhibitor-2: Plasminogen Activator Inhibitor Type 2: Minactivin: PAI-2

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category*	Citation of Document,[11] with indication,[12] where appropriate, of the relevant passages	Relevant to Claim No 13
A	AU,A, 71655/87 (BIOTECHNOLOGY AUSTRALIA PTY) 24 September 1987 (24.09.87)	1 to 13, 17 to 22
A	DE,A, 3722673 (BEHRINGWERKE AG) 19 January 1989 (19.01.89)	1 to 13, 17 to 22
A	Protein Engineering, Volume 2, No. 8, issued 1989 (IRL Press, New York) Haigwood N L et al "Variants of Human Tissue-Type Plasminogen Activator substituted at the protease cleavage site and glycosylation site, and truncated at the N-and C-Termini", see pages 615 to 619	1 to 13, 17 to 22

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
3 April 1991 (03.04.91)	11 April 1991
International Searching Authority	**Signature of Authorized Officer**
Australian Patent Office	J H CHAN

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V.[] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE 1

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [] Claim numbers ..., because they relate to subject matter not required to be searched by this Authority, namely:

2. [] Claim numbers ..., because they relate to parts of the international application that do comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [] Claim numbers ..., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4 (a):

VI.[] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING 2

This International Searching Authority found multiple inventions in this international application as follows:

1. [] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. [] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[] The additional search fees were accompanied by applicant's protest.
[] No protest accompanied the payment of additional search fees.